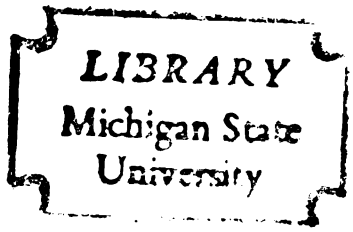

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STUDIES ON THERMOTAXIS BY PSEUDOPLASMODIA
OF DICTYOSTELIUM DISCOIDEUM

presented by

Bruce Davidson Whitaker

has been accepted towards fulfillment
of the requirements for

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Major professor

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STUDIES ON THERMOTAXIS BY PSEUDOPLASMODIA OF DICTYOSTELIUM DISCOIDEUM

By

Bruce Davidson Whitaker

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STUDIES ON THERMOTAXIS BY PSEUDOPLASMODIA OF DICTYOSTELIUM DISCOIDEUM

By

Bruce Davidson Whitaker

The degree of directed migration by pseudoplasmodia of Dictyostelium discoideum on different temperature gradients has been measured as a function of the temperature of growth and development. Pseudoplasmodia migrate toward the cooler side of a temperature gradient (negative thermotaxis) at mid-gradient temperatures > 2° below the growth/developmental temperature, and toward the warmer side of the gradient (positive thermotaxis) at higher mid-gradient temperatures. The zero-transition between positive and negative thermotaxis is dependent on the temperature of growth and development, being 14.5°, 17°, 20.5°, and 23.5° for growth/development temperatures of 16°, 18°, 23.5°, and 27.5°, respectively. For a growth/development temperature of 23.5°, the zero-transition occurs at 20.5° for pseudoplasmodia on 0.22°C/cm gradients and at 20° on 0.11°C/cm gradients. For a growth/development temperature of 18°, the zero-transition occurs at 17° for pseudoplasmodia on 0.22°C/cm gradients and at 16° on 0.11°C/cm gradients. Three thermosensors have been postulated for Dictyostelium to account for both positive and negative thermotaxis, and the adaptive zero-transition temperature between these opposite

responses -- a minimum of two thermosensors controlling positive and negative thermotaxis, plus one sensor regulating thermal adaptation. The shift of the zero-transition between positive and negative thermotaxis on 0.11°C/cm versus 0.22°C/cm gradients may indicate different temperature dependencies for the opposite responses, and thus supports a model involving a separate thermosensor for each.

Several lines of experimentation were persued to determine more about the thermosensor for thermal adaptation. Significant adaptation of thermosensing was demonstrated after 4 hours at a new temperature. The period of greatest thermal adaptation falls between the last 2-6 hours of pseudoplasmodium development (when pseudoplasmodia are formed from tight aggregates). De novo synthesis of protein or lipid appears unlikely as a mechanism for thermal adaptation during development. Evidence for the involvement of membrane lipids in the determination of the zero-transition between positive and negative thermotaxis was sought. An increase in the ratio of dienoic to monoenoic fatty acids in phospholipids from pseudoplasmodial amoebae was shown for 18° versus 23.5° pseudoplasmodia, suggesting that Dictyostelium adapts to a lower growth/developmental temperature by raising the level of fatty acid unsaturation (as do many other microorganisms). Growth of D. discoideum, NC-4, amoebae in association with bacteria of modified fatty acid composition produced a slight shift of the zero-transition between positive and negative thermotaxis. Inhibitor studies provided no evidence for a role of sterols in

thermosensory transduction in D. discoideum. Based on these data, a model has been proposed involving the Δ-5 fatty acid desaturase of Dictyostelium as the thermosensor for thermal adaptation.

DEDICATION

This thesis is dedicated to Mr. Ochs, who defined for me the term "behavior"; to Dr. Paul Biebel, who warned me of over-confidence; and to Kurt Vonnegut, Jr., who wrote:

> Tiger got to hunt,
> Bird got to fly;
> Man got to sit and wonder, "Why, why, why?"
>
> Tiger got to sleep,
> Bird got to land;
> Man got to tell himself he understand.

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Amo-1618	2-isopropyl-4-dimethylamino-5-methyl phenyl-1-piperidine-carboxylate methyl chloride
cAMP	3', 5'-cyclic adenosine monophosphate
CCC	(2-chloroethyl) trimethyl ammonium chloride
DMF	Dimethyl formamide
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
EPR	Electron paramagnetic resonance
GC-MS	Gas chromatography - mass spectrometry
GLC	Gas-liquid chromatography
LPC	Lysophosphatidyl choline
LPE	Lysophosphatidyl ethanolamine
SK&F 7997	Tris-(2-diethylaminoethyl) phosphate trihydrochloride
TLC	Thin-layer chromatography
UV	Ultraviolet

INTRODUCTION

The cellular slime mold Dictyostelium discoideum is one of the simplest eukaryotic organisms known. In spite of this simplicity the organism displays a variety of responses to sensory stimuli in its environment, both in the unicellular and multicellular stages of its life cycle. Because of these capabilities D. discoideum has been selected as a model aneural organism for the study of sensory transduction.

The Life Cycle:

In nature unicellular myxamoebae of Dictyostelium feed on soil bacteria in the leaf mulch of the forest floor. Vegetative amoebae are thought to locate the bacteria on which they feed through chemotaxis in response to secreted folic acid.[1,2] When the food supply is depleted, starvation triggers the onset of a developmental regime. The first step in development entails the aggregation of individual amoebae via chemotaxis toward pulses of cyclic adenosine monophosphate (cAMP).[3,4] The process whereby large fields of myxamoebae are entrained by an aggregation center is complex. It involves the sequential acquisition of three developmentally regulated competences: 1) the competence for chemotaxis in response to a gradient of cyclic AMP; 2) the ability to signal relay (to synthesize and release an amplified burst of cAMP in response

to a pulse of exogenous cAMP); and 3) the capacity, acquired by a small percentage of the total cell population, for the autonomous, periodic production and pulsatile release of cAMP.[5-7] After 8-9 hrs of starvation, when all three competences have arisen, aggregation is initiated by the cells autonomously releasing cAMP.[5,7] The nearest neighbors to these cells signal relay to outlying amoebae, then proceed with a step of inward chemotactic movement.[8-10] In this manner concentric or spiral waves of aggregating amoebae are formed which periodically move towards a central loose aggregate.[8,11,12] The waves are eventually supplanted by branched streams of amoebae linked end to end which radiate out from the organizing center.[8,11]

At the completion of aggregation the loose multicellular masses tighten and begin to secrete a surface slime sheath.[13,14] When a mound of cells or conus is completely covered by slime sheath it begins to erect a vertical, fingerlike projection. This structure topples over to yield a slug or pseudoplasmodium, the first of two multicellular stages of the Dictyostelium life cycle.[15] The pseudoplasmodia are capable of migration at a rate of $\simeq$ 1 mm/hr.[16,17] During the course of migration new slime sheath is continuously secreted, while the collapsed tube of old sheath material is left behind the organism as a slime trail.[18] The duration of slug migration is determined to a large extent by environmental parameters such as humidity, temperature, illumination and the pH and ionic strength of the substratum.[15,19] Ammonia appears to be one diffusable factor which governs how long slugs continue to migrate.[20,21]

At the end of migration a pseudoplasmodium culminates to form a fruiting body or sorocarp. This second multicellular stage of the life cycle consists of a vertical cellulosic stalk supporting a spore sac or sorus.[22] The pattern of cell differentiation is invariably the same during the formation of a sorocarp from a pseudoplasmodium.[22,23] The basal disc of the stalk arises from a small number of cells located at the extreme posterior end of a pseudoplasmodium. The stalk itself is composed of a cellulosic cylinder surrounding highly vacuolated, enlarged stalk cells. These originate from the anterior ≃ 20% of a slug. The remaining posterior 70-80% of the slug cells differentiate to form spores.[24-26] Spores are dispersed by wind, rain or insects, and germinate once in a more favorable environment to yield vegetative amoebae,[27,28] thus reinitiating the life cycle.

Sensory Transduction:

Sensory transduction in Dictyostelium has received little attention relative to the processes of development and differentiation. The most intensively studied sensory phenomenon exhibited by the organism is that of chemotaxis in response to pulses of cyclic AMP,[29,30] an intrinsic aspect of cell aggregation at the start of development. The second instance of chemosensory transduction by individual amoebae is the chemotactic response to folic acid secreted by food bacteria, as mentioned above. This response is expressed during the vegetative phase of the life cycle, and subsequently diminishes as cells enter the aggregation phase.[31]

Other sensory responses exhibited by Dictyostelium include phototaxis and thermotaxis by pseudoplasmodia, both of which were initially described by Raper nearly forty years ago.[15] Bonner and co-workers were the first to attempt to quantitate and further characterize these two phenomena.[32] The positive thermotactic response was found to be extremely sensitive, a pseudoplasmodium being capable of "measuring" ≃ 0.0005°C across its 100μ diameter. A crude "action spectrum" for the phototactic response provided no indication of a region in the visible spectrum which does not induce phototaxis. Based on this observation, it was concluded that phototaxis by pseudoplasmodia is merely a special case of thermotaxis, where light absorbed by one side of the pseudoplasmodium results in a localized heating and resultant thermal gradient to which the organism responds thermotactically.

Subsequent to this early investigation, several lines of evidence have clearly shown that phototaxis and thermotaxis by pseudoplasmodia of D. discoideum are mediated by separate sensory transducers. An action spectrum for phototaxis obtained by Francis[33] shows a maximum in the blue region at ≃ 440nm and a second, broad maximum in the green region centered at ≃ 550nm. The measurements of phototaxis by Bonner's group were probably performed under saturating light conditions with inadequate light filtering. Francis found pseudoplasmodia to be responsive to extremely low light intensities (≃ 0.1 to 0.3μWcm^{-2}).[33] Separate calculations by Gamble, Francis and Poff indicate that the lowest intensity of visible light which evokes slug phototaxis is well below the threshold required to

produce sufficient heating of a slug to elicit a thermotactic response.[33-35] Furthermore, it is known that the slug body serves as a convex lens during phototaxis, focusing light on the distal side where the ultimate result of increased light absorbance is an increase in slug migration rate.[33,36] Thus, when a vertical microbeam of visible light is focused on one side of the anterior tip of a migrating pseudoplasmodium, the rate of movement is increased on the side irradiated and the slug turns away from the beam.[33,36] Alternatively, when one side of a slug tip is irradiated with a vertical microbeam of infra-red radiation (which is inactive for phototaxis but produces heating resulting in thermotaxis), the organism turns in the direction of the irradiated side.

Light-induced absorbance changes associated with phototaxis have been measured in whole cells and cell homogenates of Dictyostelium discoideum, strain A_3.[37] Using these absorbance changes as a photoassay, Poff and co-workers were able to isolate and purify a pigment believed to mediate phototaxis by pseudoplasmodia.[38] The photoreceptor pigment, dubbed "phototaxin", was characterized as a high spin heme protein which undergoes a photo-oxidation in the light.[39] Phototaxin was most abundant in the mitochondrial fraction (12K pellet), and could be solublized by sonication.[37,38] However, after solublization of phototaxin the photo-oxidized pigment was no longer re-reduced in darkness.[38] It is unknown how phototaxin directs the phototactic response by pseudoplasmodia of D. discoideum. Increased synthesis and extrusion of slime sheath on the distal side of a slug where lateral

light is focused has been suggested as the mechanism whereby migration rate is increased and a phototactic turn results.[36]

There had been no evidence for either phototactic or thermotactic behavior by individual slime mold amoebae prior to the recent data of Häder and Poff.[32,40-43] Photoaccumulation of pre-aggregation phase Dictyostelium amoebae in a low intensity light field was demonstrated using a light trapping technique devised for the study of photomovements by blue-green algae.[42] At higher light intensities the amoebae were observed to disperse from the light trap.[43] Through the use of a microscope-video recorder system, these opposite photo-responses were further shown to be attributable to either positive or negative phototaxis by individual amoebae. The action spectrum for amoebal phototaxis derived from the light trap experiments was distinctly different from that of slug phototaxis. In particular, there was a maximum in the red at ≃ 640nm for the photoresponses of amoebae, whereas phototaxis by pseudoplasmodia drops off quickly after ≃ 610nm.[33,37,42,43] The mutant strain of Dictyostelium, PT_3, which produces non-phototactic pseudoplasmodia, exhibited thresholds for photoaccumulation and photodispersal an order of magnitude greater than amoebae of the wild type strain, NC-4.[44] This may imply that phototaxis by amoebae and pseudoplasmodia involves a common pathway. However, the possibility that slug phototaxis is the result of phototaxis by the individual amoebae comprising the multicellular structure is remote. Pseudoplasmodia are responsive to light intensities at least two orders of magnitude below the threshold for photoaccumulation of amoebae.[45]

Furthermore, negative phototaxis by pseudoplasmodia at relatively high light intensities does not occur.

Light trap experiments testing wavelengths in the near infra-red have also been performed. There was no evidence for either accumulation or dispersal of pre-aggregation phase amoebae in response to infra-red radiation over three decades of intensity.[42,46] These data corroborate the earlier report by Bonner that individual myxamoebae are non-thermotactic.[32] Thus, thermotaxis by a pseudoplasmodium must be a function of the entire, multicellular structure. Loomis has proposed that the surface sheath might serve in the control of thermotaxis through a slight decrease in the relative humidity, and thus elasticity, of the sheath on the warmer side of a pseudoplasmodium.[47] He argued that over a span of hours such small differences in the elasticity of the sheath could ultimately result in a positive thermotactic turn. Poff and Skokut have refuted this model on the basis of the narrow temperature range over which pseudoplasmodia are positively thermotactic.[35] A thermal effect on the hydration of the slime sheath should display no such strong temperature dependence.[48] Similarly, the possibility that positive thermotaxis is the result of a general thermal effect on metabolic rates was ruled out with the demonstration that the overall rate of slug migration is relatively temperature independent over a broad temperature range. These data led Poff and Skokut to conclude that there must be a specific "biothermometer" involved in thermosensory transduction in Dictyostelium.[35]

The Biothermometer for Thermotaxis:

It is well documented that patches of lipid in biomembranes undergo abrupt phase transitions from the gel to liquid-crystalline state within the physiological temperature range of the cell.[49-51] The change in membrane fluidity resulting from a phase transition, as well as membrane lipid composition in general, have a profound effect on membrane function.[52-54] It is also known that a variety of microorganisms adjust to a new growth temperature by modifying the lipid composition of their membranes, thereby maintaining an optimum membrane fluidity.[55-58] The fact that the low temperature limit for positive thermotaxis by pseudoplasmodia of D. discoideum could be shifted down by lowering the growth temperature of the amoebae suggested to Poff and Skokut that a membrane lipid or lipids might serve as the hypothetical biothermometer.[35] The data of Poff and Skokut also favor a spatial rather than temporal mechanism of temperature measurement in Dictyostelium.[35] A pseudoplasmodium must therefore be capable of sensing a very small temperature differential across its width. A phase transition could act as a high gain amplifier for the measurement of small temperature differences. Furthermore, a change in membrane lipid constituents resulting from a change in growth temperature would be expected to shift the temperature of the phase transition, and thus the termosensory range for positive thermotaxis as well. Based on these criteria, Poff and Skokut proposed that the thermosensory range for positive thermotaxis is centered upon, and governed by, a membrane lipid phase transition.[35]

The work encompassed by this thesis was intended to test the "phase transition-as-biothermometer" hypothesis with several different approaches. The first approach was to seek biochemical evidence for "homeoviscous adaptation" in Dictyostelium, i.e. a change in cellular lipid composition induced by a change in the temperature of growth and/or development.[56] Specifically, changes in the level of desaturation of the fatty acid profile, as well as differences in the amount of free and esterified sterols, were sought using gas-liquid chromatography and gas chromatography/mass spectrometry (GLC and GC-MS). Turnover of cellular lipids associated with pseudoplasmodium formation was also studied.

A second series of experiments sought to disrupt the normal thermotactic response of pseudoplasmodia through perturbations of the cell membrane. Free sterols are known to play an important role in membrane fluidity and in the sharpness of lipid phase transitions due to their interactions with phospholipids.[50,59-61] Hence, a variety of inhibitors of sterol biosynthesis and function were tested for their ability to shift the thermosensory range of thermotaxis or to eliminate the thermotactic response altogether. Fatty acid supplementation experiments were also tried in an attempt to radically alter the fatty acid composition of cellular membranes,[62,63] and thereby perturb the thermotactic response.

The third line of investigation was intended as an expansion of the work of Poff and Skokut[35] on thermal adaptation of the thermosensory range for positive thermotaxis. The phase transition/biothermometer model predicted that the narrow thermosensory range for

positive thermotaxis should be shifted up or down in accordance with a raising or lowering of the temperature of growth and development. Therefore, a comparison of the thermosensory range of 18°C versus 23.5°C pseudoplasmodia* was carried out on 0.22°C/cm gradients with midpoints extending from 15° → 29°C. These experiments resulted in the observation of previously unreported "negative" thermotaxis by pseudoplasmodia on thermal gradients with a midpoint temperature $\geq$ 1° → 4°C below the temperature of growth and development. This finding necessitated a reappraisal of the possible role of a membrane phase transition in thermosensing by pseudoplasmodia. Studies of the rate and developmental timing of thermal adaptation of the thermotactic range for Dictyostelium were also performed.

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*For the sake of brevity, the convention of using "T°C pseudoplasmodia" to indicate that these pseudoplasmodia were allowed to form at T°C from amoebae grown at T°C has been adopted throughout this thesis.

SECTION I

HOMEOVISCOUS ADAPTATION AND THERMAL ADAPTATION OF THERMOSENSING IN *DICTYOSTELIUM DISCOIDEUM*

INTRODUCTION:

A variety of micro-organisms including bacteria, mycoplasmas, algae and yeasts, as well as some higher plants and animals, appear to utilize the mechanism of "homeoviscous adaptation" to cope with changing environmental temperature.[55,57,58,64-69] Homeoviscous adaptation is a term coined by Sinensky[56] to describe the process through which bacteria alter the fatty acid composition of membrane lipids in response to a change of growth temperature, and thereby maintain a constant membrane viscosity. The viscosity of the cell membrane has been shown to be an important aspect of membrane function, affecting functional parameters such as differential permeability and the activity of integral membrane-bound enzymes.[52-54,70]

The change brought about by decreasing growth temperature in membrane phospholipids of *E. coli* and other micro-organisms is an increase in the proportions of unsaturated and short chain fatty acids.[55-58,64,65] Raising the ratio of unsaturated to saturated fatty acids in a phospholipid bilayer reduces the degree of ordering of the hydrocarbon "tails".[49,71,72] The phospholipids are less tightly packed together and thus, at a given temperature, the

bilayer is more fluid.[49,51,72] In addition, there is a reduction in the temperatures at which phospholipid solid-to-liquid-crystalline phase transitions occur.[49,51,72] It is this corollary of homeoviscous adaptation, the shift of membrane phase transition temperatures, which is pertinent to the current hypothesis of the "biothermometer" for positive thermotaxis by pseudoplasmodia of Dictyostelium discoideum.

Poff and Skokut observed a downward shift of the low temperature limit for positive thermotaxis in Dictyostelium when the growth temperature was lowered.[35] This observation was consistent with the hypothesis that the thermosensory range for positive thermotaxis is governed by a membrane lipid phase transition, and that the phase transition/biothermometer changes in accordance with the growth temperature via homeoviscous adaptation. Although the demonstration of homeoviscous adaptation in Dictyostelium would not prove the role of a membrane lipid phase transition as the biothermometer for thermotaxis, it could at least be considered as good supportive evidence. Therefore, a study of the effect of growth and developmental temperature on the lipid composition of D. discoideum was undertaken.

Four types of experiments were carried out to establish whether homeoviscous adaptation is evident in Dictyostelium. The first set of experiments involved the comparison of fatty acid composition in three membrane fractions from lysed vegetative amoebae of D. discoideum, NC-4, grown at either 18° or 23.5°C. The second set of experiments was directed toward the question of

how the periods of starvation and pseudoplasmodium formation following vegetative growth affect the fatty acid composition of Dictyostelium amoebae. A comparison was made of the fatty acyl methyl ester profiles of total lipids from: (1) vegetative, (2) starved, and (3) pseudoplasmodial amoebae which were grown, and in the case of the latter two, incubated at either 18° or 23.5°C. The third set of experiments was performed to provide a more complete characterization of all the lipid constituents found in young, migrating (20-23 hr) pseudoplasmodial amoebae which were grown and allowed to develop at either 18° or 23.5°C. The fourth and final investigation utilized the technique of gas chromatography/mass spectrometry (GC-MS) in a test of one possible mechanism whereby homeoviscous adaptation in Dictyostelium might arise.

MATERIALS AND METHODS:

1. Membrane isolation and fatty acid analysis:

Cultures: Dictyostelium discoideum strain NC-4 was grown on Sussman's medium nutrient agar plates at either 18° or 23.5°C in association with the bacterium Klebsiella aerogenes according to the method of Sussman.[73] Individual nutrient agar plates were inoculated with 0.7 ml of a bacterial culture grown for 24-48 hours at 23.5°C on Bactopeptone (Difco) HL-5 broth medium.[74] Spores of NC-4 were collected from sorocarps with a small, flame-sterilized loop, and one loopful of spores was mixed with the bacterial suspension on each plate. The suspension was then spread evenly over the agar surface with a flamed, bent glass rod. Within 24 hrs a dense lawn of the bacterial associate formed, and by 48 hrs the slime mold amoebae had cleared the bacteria from much of the agar surface. At this time vegetative amoebae were harvested by flooding the plates with 5 ml of ice cold distilled water, rubbing the entire agar surface with an index firger, and pouring off the cell suspension into a 50 ml Nalgene centrifuge tube. Amoebae from approximately 50 plates were pelleted by centrifugation at 750x g for 2 min, and freed of bacteria by washing 3x in cold phosphate salts buffer (1.5g KCL, 0.5g $MgCl_2$, 1.6g K_2HPO_4, 1.8g KH_2PO_4 and 0.5g streptomycin sulfate per liter; pH 6.5).

Cell lysis and membrane isolation: The washed pellet of vegetative amoebae grown at either 18° or 23.5°C was resuspended at 2 x 10^8 cells/ml in cold phosphate salts buffer containing 1μM phenylmethyl sulfonyl fluoride (Sigma Chemical Co.), a selective protease

inhibitor.[75] Cells were broken in a 50 ml Dounce homogenizer (Kontes Glass) with a tight-fitting pestle at 0°C. Between 50-70 strokes were usually required to achieve 70-80% breakage.

A plasma membrane-enriched fraction was obtained using the two-phase polymer system of Brunette and Till.[76] The cell homogenate was centrifuged at 650x g for 5 min to pellet whole cells and large membranes. The pellet was then resuspended in 15 ml of cold upper phase solution containing Dextran T-500 (Pharmacia Chemical Co.). This suspension was mixed thoroughly with 15 ml of lower phase solution containing polyethylene glycol 6000 (J.T. Baker Chemical Co.). The mixture was centrifuged at 10,000x g for 10 min to separate the two phases. Large membranes which accumulated at the phase interface were recovered with a Pasteur pipet. The membrane suspension was diluted with two volumes of cold 0.32% sodium citrate buffer containing 0.04% Na_4EDTA and 5mM $MgCl_2$, and centrifuged at 10,000x g for 10 min. The membrane pellet was washed twice and resuspended in 0.5 ml of citrate buffer.

Additional membranes were collected from the low speed supernate of the cell homogenates by centrifugation at 31,000x g for 20 min. The high speed supernate was removed by pipetting. Using a Pasteur pipet, the loosely packed, opalescent membrane pellet was gently rinsed from atop the firmly packed, red-brown mitochondrial pellet with a few milliliters of citrate buffer. The membrane suspension was then removed with a Pasteur pipet and centrifuged for 10 min at 10,000x g to pellet the larger membrane fragments. The pellet was washed twice and resuspended in 1.5 ml of citrate buffer.

Renografin density gradient centrifugation of membranes: Both the plasma membrane-enriched and crude membrane fractions were separated into several distinct bands on linear Renografin density gradients by a modification of the method of McMahon et al.[77] Linear density gradients from 0-38% Renografin 76 (E.R. Squibb) were prepared in 9/16" x 3½" cellulose nitrate tubes (Beckman Co.) using a standard two-chambered gradient mixer. The Renografin density gradient buffer contained 0.32% sodium citrate, 0.04% Na_4EDTA and 5mM $MgCl_2$. For each batch of membranes, four Renografin density gradients were prepared and left in the refrigerator for one hour before use. The 0.5 ml membrane suspension obtained from the two phase isolation system was freed of clumps with several strokes in a 1.0 ml Dounce homogenizer (Kontes Glass) and carefully layered on top of one density gradient with a Pasteur pipet. The 1.5 ml crude membrane suspension was freed of clumps in the same manner and 0.5 ml was layered on top of each of the remaining three density gradients. The four gradients were then centrifuged for 15 hrs in an SW-40 rotor at 23,000 rpm in a Beckman L2-65B preparative ultracentrifuge at 4°C. After centrifugation, the four membrane bands depicted in Figure 1 were recovered with a 9" Pasteur pipet drawn to a fine tip over a Bunsen flame. Identical bands from each gradient were pooled, diluted with two volumes of citrate buffer and centrifuged at 31,000x g for 20 min. The membrane pellets were washed twice more with citrate buffer and collected in Corex glass centrifugation tubes prior to lipid extraction.

Total lipid extraction: Total lipid was extracted from the pelleted membrane fractions with 20 volumes of chloroform-methanol (2:1).[78]

(All solvents used in lipid extraction were glass-distilled.) The membrane pellet in a Corex tube was suspended in methanol by vortexing for 10 sec, and the suspension transferred to a screw-cap culture tube with a Pasteur pipet. The Corex tube was then rinsed with twice the volume of chloroform, and the chloroform rinse added to the methanol-membrane suspension. The culture tube was flushed with nitrogen and quickly sealed with a Teflon-lined cap. Total membrane lipids were extracted overnight at room temperature. The lipid extract was cleared of particulate membrane debris by centrifugation at 1000x g for 5 min, and transferred to a screw-cap conical centrifuge tube with a fine-tipped Pasteur pipet. A Folch wash[78] was then performed to remove non-lipid contaminants. One quarter of the total liquid volume of 0.8% (w/v) KCl solution was added to the lipid extract, and the conical centrifuge tube was flushed with nitrogen and sealed with a Teflon-lined cap. The tube was vortexed for several seconds and then centrifuged at 750x g for 2 min to separate the phases. The upper methanol-water phase was removed with a fine-tipped Pasteur pipet and discarded. The lipid extract was then given a second wash with 1/4 the total liquid volume of distilled water-methanol (1:1). After separation of the phases by centrifugation, the chloroform lipid extract was removed with a fine-tipped Pasteur pipet and dried over anhydrous Na_2SO_4. Chloroform was evaporated from the purified lipids under a stream of nitrogen while being warmed at 40°C.

Transesterification of total lipid: Methyl esters of fatty acids from the membrane total lipid were routinely prepared by

transesterification with 0.5N sodium methoxide in dry methanol, following the procedure of Luddy et al.[79] Fresh reagent was prepared by disolving 0.7g of finely-chopped, clean sodium metal in 62 ml of dry methanol. Lipid samples to be transesterified were suspended in a small volume of benzene in a micro-reaction vial (Supelco). An equal volume of 0.5N sodium methoxide reagent was added, the vial tightly capped, and the mixture heated in a temperature block at 80°C for 20 min. After cooling, the reaction was terminated by the addition of 1.5 volumes of distilled water, followed by the addition of 1.5 volumes of diethyl ether. The ether phase containing the fatty acyl methyl esters was washed once with an equal volume of distilled water, dried over anhydrous Na_2SO_4 and evaporated under a stream of nitrogen to remove traces of benzene. The fatty acyl methyl esters were then resuspended in a small volume of ether for GLC analysis.

Gas-liquid chromatography of fatty acyl methyl esters: Fatty acyl methyl esters were separated and quantified by gas-liquid chromatography (GLC). Separations were performed on a Varian model 3740 gas chromatograph. Flame ionization detectors and 6 ft glass coil columns (1/4" o.d.; 2mm i.d.) were used for all GLC work. The cyanosilicone packing series of 10% SP-2300, SP-2330 and SP-2340 (Supelco, Inc.) was chosen for the separation of fatty acyl methyl esters. Isothermal column oven temperatures between 180° and 200°C, and nitrogen carrier flow rates of 20cc to 30cc/min were used to obtain the best separations with each of the three column packings. Identification of methyl esters of Dictyostelium fatty acids was

based on retention times on the three packing materials compared with authentic standards, and on the fatty acid profiles for Dictyostelium published in the literature.[80-82] Comparison with the fatty acyl methyl esters prepared from lipids of Klebsiella aerogenes was also helpful in the identification of bacterial cyclopropane fatty acids incorporated into lipids of Dictyostelium. The relative percentages of individual fatty acyl methyl esters in the profiles from Dictyostelium lipids were computed on an area percent basis. Peak area integrations and area percent calculations were performed by a Varian CDS-111 chromatography data system. Fatty acyl methyl ester standards were purchased from Sigma Chemical Co., Supelco and Applied Science Laboratories.

2. Homeoviscous adaptation - the effects of starvation and development:

Cultures: Amoebae of D. discoideum, NC-4, were grown in suspension culture in association with the bacterium Klebsiella aerogenes using a modification of a method described by Sussman.[73] Cultures of K. aerogenes were grown on Bactopeptone (Difco)-HL-5 broth medium[74] at 23.5°C, and harvested at late log phase (36-48 hrs) by centrifugation (7000x g, 5 min). The pellet was washed twice in cold potassium phosphate buffer, pH 6.0, and once in cold distilled water. The bacteria were then resuspended in a small volume of cold distilled water, quick frozen with liquid nitrogen and lyophilized. Dried K. aerogenes were added at a concentration of 2mg/ml to sterile 15mM potassium phosphate buffer (pH 6.0) prior to inoculation with Dictyostelium amoebae. The slime mold cultures were agitated on a rotary shaker (New Brunswick Scientific Co.) at 250 rpm and were

harvested at the late log phase of growth (32-48 hrs) after reaching a titer of 8×10^6 to 1.5×10^7 cells/ml. Vegetative amoebae were freed of bacteria by twice suspending in cold 15mM potassium phosphate buffer, pH 6.0, and pelleting at 750x g for 2 min. The washed amoebae were: 1) resuspended in cold distilled water and centrifuged (750x g, 2 min) in a Corex tube, followed by lipid extraction, 2) resuspended in phosphate buffer at 10^7 cells/ml in an Erlenmeyer flask and placed on a rotary shaker at the temperature of growth for 16 hrs starvation (clumping, but no development occurs under these conditions) or 3) resuspended in cold distilled water at 10^8 cells/ml and spread evenly with a bent glass rod on 2% water agar Petri plates, which were wrapped in foil and incubated at the temperature of growth for either 16 or 34 hrs. Amoebae starved in phosphate buffer were harvested by centrifugation, washed once in cold distilled water, pelleted in a Corex tube (750x g, 2 min) and extracted. Newly formed (16 hr incubation) and "depleted" (34 hr incubation) pseudoplasmodia were recovered from the agar surface of the Petri plates by scraping with a bent glass rod. The harvested pseudoplasmodia were suspended in cold distilled water and pelleted by centrifugation at 6000x g for 5 min. Intact pseudoplasmodia were disrupted by repeated, vigorous pipetting in a few milliliters of distilled water with a fine-bore 5 ml pipet. Pseudoplasmodial amoebae were separated from slime sheath by repeated (3-4x) low speed centrifugation (750x g, 1 min) and resuspension in cold distilled water. The final wash prior to lipid extraction was performed in a Corex tube.

Lipid extraction: Total cellular lipid was extracted from 18° and 23.5°C vegetative, starved or pseudoplasmodial amoebae with 20x the packed cell volume of a solvent mixture composed of isopropanol-methanol-chloroform, 1:1:4, as follows: One volume of hot isopropanol was added to a Corex centrifuge tube containing the pellet of washed amoebae and the tube was vortexed for 10 sec. One volume of methanol was added, and the cell suspension transferred to a screw cap culture tube. The Corex tube was rinsed with four volumes of chloroform which were then added to the isopropanol-methanol extract.[83,84] The culture tube was flushed with nitrogen and quickly sealed with a Teflon-lined cap. The total cell lipids were extracted overnight at room temperature. The solvent lipid extract was separated from a small aqueous phase containing the cell debris by centrifugation at 1000x g for 5 min. A fine-tipped Pasteur pipet was used to transfer the lipid extract to a sintered glass funnel with a filtering funnel of the same diameter taped to the top to form an enclosed chamber. Nitrogen under pressure was applied through the stem of the filtering funnel, forcing the lipid extract through the sintered glass funnel and into a screw cap conical centrifuge tube. The lipid extract was then washed by the Folch procedure,[78] dried over anhydrous Na_2SO_4 and the solvent driven off with nitrogen as described in M & M section I.1.

Transesterification and GLC analysis: Total cell lipids were transesterified with 0.5N sodium methoxide and the resultant fatty acyl methyl esters were analyzed by GLC as described in M & M section I.1.

3. Complete characterization of lipids from pseudoplasmodial amoebae:

Cultures: Two liter suspension cultures of D. discoideum, NC-4, which reached titers of 7 x 10^6 and 1 x 10^7 cells/ml at 23.5° and 18°, respectively, were grown, harvested by centrifugation and freed of bacteria as described in M & M section I.2. The pellets after the final wash were resuspended in cold distilled water at 10^8 cells/ml and spread with a bent glass rod over the surface of 2% water agar coating the bottom of large (12" x 15" x 3") plastic trays. The tray tops were covered with heavy foil and each tray draped with black cloth (pseudoplasmodia culminate when exposed to overhead illumination).[19] The covered trays containing either 23.5° or 18° grown amoebae were incubated in darkness at the temperature of growth for 20 or 23 hr, respectively. After development, the pseudoplasmodia were harvested, disrupted and the amoebae freed of slime sheath as described in M & M section I.2.

Lipid extraction and fractionation: Total lipid was extracted from the washed, pelleted pseudoplasmodial amoebae, given a Folch wash, dried over anhydrous Na_2SO_4 and the solvent driven off with nitrogen as described in M & M section I.2. The purified total lipids from 18° and 23.5° pseudoplasmodial amoebae were fractionated into neutral lipids, glycolipids and phospholipids by silicic acid column chromatography.[85] The columns were prepared by pipetting a slurry of 0.4g silicic acid (325 mesh, Fisher Chemical Co.) in 2.5 ml of chloroform into a 6" Pasteur pipet plugged with glass wool. After settling, the bed of silicic acid was washed with 5 ml of chloroform. The entire batch of lipid (≃ 20-40 mg) was then taken up in 0.5 ml

of chloroform and pipetted over the bed of silicic acid. All of the lipid solution was allowed to run onto the column before adding more solvent. Due to the extremely fine particle size of the silicic acid used, it was necessary to force solvents through the column with nitrogen under pressure. The flow rate attained was ≃ 0.1 ml/min. Neutral lipids were eluted with 7 ml of chloroform, followed by elution of glycolipids with 5 ml of acetone and finally, phospholipids were eluted with 7 ml of methanol. The three lipid fractions were freed of solvent under a stream of nitrogen and stored under liquid nitrogen prior to transesterification and/or thin-layer chromatography.

Thin-layer chromatographic separations of neutral lipids and phospholipids were carried out on 20 x 20 cm glass plates precoated with a 250μ film of silica gel GF (Analtech Laboratories). Neutral lipids in chloroform and phospholipids in chloroform-methanol (2:1) were spotted with a 5μl Hamilton syringe in 2 cm wide bands located 3 cm from the base of the plate. One dimensional TLC separations of the phospholipid and neutral lipid fractions prepared by silicic acid column chromatography were carried out in solvent mixtures of chloroform-methanol-acetic acid-water (85:15:10:3.5)[86] and benzene-diethyl ether-ethyl acetate-acetic acid (80:10:10:0.2),[87] respectively.

Derivatization of lipids and quantification by GLC: Phospholipids, glycolipids and steryl esters were transesterified with 0.5N methanolic sodium methoxide reagent and the resultant fatty acyl methyl esters quantified by GLC as described in M & M section I.1.

Free sterols were derivatized to steryl acetates for GLC analysis by suspending in 1.0 ml of acetic anhydride (Mallinkrodt Chemical Co.) in a micro-reaction vial and heating the vial in a temperature block at 140°C for 30 min to yield a clear solution.[88] The steryl acetates were dried under a stream of nitrogen and recrystallized from hot ethanol. The recrystallized product was taken up in 0.5 ml of chloroform for GLC analysis. Steryl acetates were separated on 3% SE-30 (Supelco, Inc.) packed in a 6 ft glass coil column (1/4" o.d., 2 mm i.d.). The separations were performed using a Varian model 3740 gas chromatograph with the column oven set at 245°C and the nitrogen carrier flow rate at 40 cc/min. Identification of sterols of Dictyostelium was based on comparison of retention times with a variety of standards and on the literature.[89-91] Steryl acetates were quantified by comparison of their peak areas with the peak areas of known amounts of internal standards, either cholesterol (Nutritional Biochemicals) or stigmasterol (Sigma Chemical Co.). Peak area integration and area percent calculations were performed by a Varian CDS-111 chromatography data system. Additional sterol standards included sitosterol (Aldrich Chemical Co.) and ergosterol (Calbiochemicals).

Identification and quantification of lipids on TLC plates: A 0.2% (w/v) solution of 2,7-dichlorofluorescein in 95% methanol (Supelco, Inc.) was used as a non-specific, non-destructive spray for the visualization of lipid spots on thin-layer chromatograms. Under UV light lipid spots appeared bright yellow in the presence of this dye, and could be recovered quantitatively by scraping the plate

and eluting the silica gel with the appropriate solvents.[85] Phospholipids were visualized by spraying a TLC plate with an acidic molybdate reagent[92] which rendered the phospholipid spots deep blue on a light blue background. Phosphatidyl choline was identified by spraying with Dragendorff's reagent,[93] and phosphatidyl ethanolamine by spraying with 0.2% ninhydrin in ethanol (Sigma Chemical Co.) and heating for several minutes at 100°C.[93] Sterols and steryl esters were identified by their characteristic rose-violet color which appears shortly after spraying with acidic 0.5% (w/v) $FeCl_3$ reagent.[94]

Phospholipids separated by TLC were visualized with 2,7-dichlorofluorescein spray and scraped from the plate into separate conical centrifuge tubes. The phospholipids were then eluted from the silica gel with 20 volumes of chloroform-methanol (2:1). Fine-tipped Pasteur pipets were used to transfer the phospholipid solutions to separate, new 13 x 100 mm Kimax culture tubes. Quantitative analysis of the total phospholipid phosphorus in each tube was carried out according to the method of Ames.[95] Solvents were evaporated from the tubes with a stream of nitrogen, and the phospholipid in each tube ashed with 10% $Mg(NO_3)_2.6H_2O$ in 95% ethanol over a Bunsen flame. Any pyrophosphate formed by the ashing procedure was hydrolyzed to phosphate by the addition of 0.5N HCl and incubation of the tubes in a boiling water bath for 15 min. After cooling, an equal volume of acidic ascorbate-ammonium molybdate reagent was added to each tube. The tubes were then incubated at 45°C for 20 min and the absorbance measured at 820nm in

a Beckman Spec 70 spectrophotometer. The total phospholipid phosphorus content of each major phospholipid fraction was obtained by comparison of the absorbance values at 820nm with a standard curve of the absorbance at 820nm by samples prepared from linearly increasing concentrations of Na_2HPO_4 solution (covering a range from 0-100nmol of PO_4). Phospholipids of Dictyostelium pseudoplasmodial amoebae separated by TLC were identified with selective spray reagents (see above) and by comparison of R_f values with authentic standards. Phospholipid standards used for co-chromatographic identification of Dictyostelium phospholipids (phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl inositol, phosphatidyl serine, phosphatidic acid, lysophosphatidyl choline and lysophophatidyl ethanolamine) were obtained from Sigma Chemical Co.

4. Heavy isotope double label experiment using GC-MS:

Cultures: Six 100 ml suspension cultures of D. discoideum, NC-4, were grown at 23.5°C, harvested and freed of bacteria with two washes in phosphate buffer as described in M & M section I.2. The washed pellet of amoebae was resuspended in 200 ml of cold distilled water and the cell suspension divided into 100 ml portions in separate centrifugation bottles. The cells were centrifuged at 750x g for 2 min and the two equal pellets resuspended at 10^7 cells/ml in either 20mM 1-^{13}C sodium acetate or 20mM 2-D_3 sodium acetate. The two cell suspensions were transferred to separate 500 ml Erlenmeyer flasks which were fitted with cotton and cheesecloth plugs and placed on a rotary shaker for 2 hrs at 23.5°C. After the 2 hr incubation the

two cell suspensions were centrifuged at 750x g for 2 min and the pellets resuspended at 10^8 cells/ml in fresh 20mM solutions of the appropriately labelled sodium acetate. The cell suspensions were then spread on 2% water agar in separate large trays, which were covered with foil and draped with black cloth. The tray containing amoebae suspended in 20mM 2-D_3 sodium acetate was incubated at 23.5°C and the tray with amoebae suspended in 1-^{13}C sodium acetate was incubated at 18°C, both for 20 hrs. At the end of incubation pseudoplasmodia from each tray were harvested and disrupted, and the pseudoplasmodial amoebae freed of slime sheath as described in M & M section I.2. The final distilled water wash was performed in Corex tubes. A control batch of amoebae was incubated at 23.5°C in the presence of 20mM unlabelled sodium acetate, but otherwise handled in precisely the same manner as the two experimentals.

Lipid extraction and fractionation: Total lipid was extracted from the three pellets of pseudoplasmodial amoebae as described in M & M section I.2. The phospholipid fraction from each of the three total lipid extracts was separated from the neutral lipids by thin-layer chromatography in the neutral lipid TLC solvent mixture as described in M & M section I.3 (phospholipids remained at the origin when plates were developed with this solvent mixture, and were recovered by scraping the plate and eluting the silica gel with chloroform-methanol, 2:1).

Analysis of fatty acyl methyl esters by GLC and GC-MS: The control (development at 23.5°C, unlabelled acetate) and two experimental (development at 23.5°C, 2-D_3 acetate and development at 18°C, 1-^{13}C

acetate) phospholipid fractions were transesterified as described in M & M section I.1. Quantitative pre-runs of esters to be analyzed by GC-MS were performed on a Hewlett-Packard model 5830A gas chromatograph, and mass spectrometric analysis was carried out using a Hewlett-Packard model 5985 mass spectrometer. For the gas chromatographic pre-runs, fatty acyl methyl esters were separated on a 6 ft glass coil column (1/4" o.d., 2 mm i.d.) packed with 10% SP-2330 (Supelco, Inc.) with the column oven at 180°C and the nitrogen carrier flow rate at 21 cc/min. The same column and nitrogen carrier flow rate were used for the gas chromatographic separation of fatty acyl methyl esters during the analysis by GC-MS, with the column oven temperature programmed to increase from 170°-185°C at a rate of 2.5°C/min. The technique of chemical ionization with isobutane was employed for the GC-MS analysis in order to increase the percentage of the molecular ion of each ester.[96]

Heavy isotope labelled compounds: 2-D_3 sodium acetate (99 atom % deuterium) and 1-^{13}C sodium acetate (90 atom % carbon-13) were purchased from Merck, Sharp and Dohme, Inc.

RESULTS:

1. Membrane isolation and fatty acid analysis

The cell membrane preparations from vegetative amoebae of D. discoideum, NC-4, grown at either 18° or 23.5°C separated into four distinct bands on Renografin linear density gradients as shown in Figure 1. The banding patterns of the crude and plasma membrane-enriched fractions were qualitatively the same. Quantitatively, on the plasma membrane-enriched gradients band B3 was very faint and the proportion of B4 was increased with respect to B1 and B2 in comparison with equivalent bands on the crude membrane gradients. In Table I the fatty acyl methyl ester profiles of bands B1, B2 and B4 are compared for vegetative amoebae grown at either 18° or 23.5°C. The fatty acyl methyl ester profile for band B3 is also presented for one temperature of growth (23.5°C). B3 was shown to be predominantly intact mitochondria by low temperature spectroscopy of the Soret and α-peaks of cytochromes in the respiratory chain. Membrane band B4 can be tentatively identified as the plasma membrane fraction, based in part on the observed increased proportion of this band on the plasma membrane-enriched gradients, in part on a Fluram (Roche Diagnostics)fluorescent labelling study of D. discoideum, Ax_2, plasma membranes on Renografin density gradients,[97] and in part on the literature.[77]

Regardless of the identity of the three membrane fractions B1, B2 and B4, the same trend is apparent in the fatty acid profile of each when equivalent fractions from 18° and 23.5° grown amoebae are compared. For 18° versus 23.5° cell membranes there is a general

Figure 1. Banding pattern of membranes from vegetative amoebae on linear Renografin density gradients.

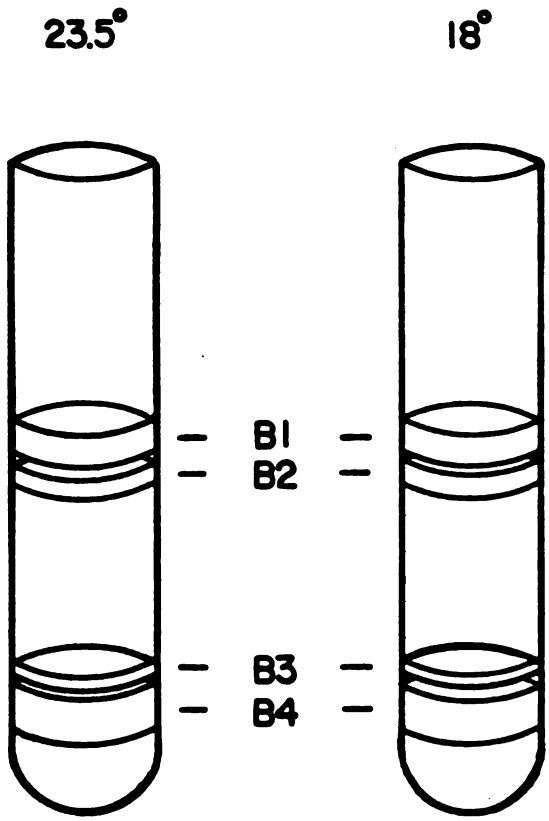

23.5°
18°
B1
B2
B3
B4

Table I. Fatty Acid Composition of Vegetative Cell Membranes

Membrane band	B1		B2		B3	B4	
Growth temp.	18°C	23.5°C	18°C	23.5°C	23.5°C	18°C	23.5°C
Fatty acyl methyl ester							
16:0	6.3	2.5	4.8	2.8	2.9	11.9	2.6
16:1(9)	2.1	2.1	2.8	1.7	3.8	0.9	1.7
16:2(5,9)	2.0	1.3	3.0	2.7	3.1	13.3	10.0
16:cyc(9,10)	9.4	9.0	6.0	9.8	12.5	6.3	12.8
18:0	0.8	0.9	0.3	1.4	1.4	4.1	4.6
18:1(11)	45.9	47.7	48.6	41.9	28.8	10.7	11.4
18:2(5,11)	21.0	17.8	27.7	19.4	33.6	48.3	46.2
18:cyc(11,12)	11.8	18.4	6.8	18.9	11.9	2.9	7.4
19:1(11)	0.5	0.3	0.1	1.2	2.0	1.5	3.2
Total	99.8%	100.0%	100.1%	99.8%	100.0%	99.9%	99.9%
% Saturated	7.1	3.4	5.1	3.2	4.3	16.0	7.2
% Monounsaturated	48.5	50.1	51.5	44.8	34.6	13.1	16.3
% Diunsaturated	23.0	19.1	3.07	22.1	36.7	61.6	56.2
% Cyclopropane	21.2	27.4	12.8	28.7	24.4	9.2	20.2

increase in the percentages of palmitate and the diunsaturated fatty acids, with a concommitant decrease in the percentages of the bacterial cyclopropane fatty acids. It is difficult to interpret what these differences mean in terms of membrane fluidity, and therefore, homeoviscous adaptation. The rise in the proportion of diunsaturated fatty acids clearly fits the "dogma", and the increase in palmitate could be viewed as a trend toward shorter fatty acyl chain length. However, the cyclopropane fatty acids are apparently quite similar to the monounsaturated fatty acids in their membrane behavior,[52] so it is possible that the fatty acid changes observed in membranes from 18° grown compared with 23.5° grown amoebae represent an even trade in terms of overall membrane fluidity. One could argue that the differences in fatty acid composition of membranes from amoebae grown at the two temperatures merely reflect changes in the fatty acid composition of bacterial lipids during the growth of the bacterial "lawn". However, total lipids from Klebsiella aerogenes grown on HL-5 broth medium at 18° had an increased proportion of cyclopropane fatty acids relative to total lipids from bacteria grown at 23.5°.

2. Homeoviscous adaptation - the effects of starvation and development:

Table II presents a comparison of the fatty acid composition of total cellular lipid from vegetative, starved and pseudoplasmodial amoebae of D. discoideum, NC-4, for the two temperatures of growth/development, 18° and 23.5°C. The differences in the fatty acid profile of vegetative cells at the two growth temperatures (the

Table II. Fatty Acid Composition of Total Cell Lipids

Fatty acyl methyl ester	Vegetative Amoebae Temperature of Growth		Starved Amoebae (16 hr) Temp. of Growth and Starvation		Pseudoplasmodial Amoebae (16 hr) Temp. of Growth and Development		Pseudoplasmodial Amoebae (34 hr) Temp. of Growth and Development	
	18°C	23.5°C	18°C	23.5°C	18°C	23.5°C	18°C	23.5°C
14:0	1.1	1.3	--	--	--	--	--	0.2
15:0	0.2	0.3	0.3	0.1	0.4	0.4	0.3	0.5
16:0	5.6	5.3	3.5	2.7	4.8	5.2	4.6	5.4
16:1(9)	6.3	7.6	3.9	4.0	3.6	4.2	4.6	4.9
16:2(5,9)	10.7	8.1	9.2	7.5	10.6	9.5	9.7	6.4
16:cyc(9,10)	18.0	21.9	7.9	6.4	4.4	7.3	6.0	3.8
18:0	1.8	1.4	1.1	1.4	1.6	1.8	0.5	1.8
18:1(11)	21.1	18.4	22.3	23.6	24.7	23.5	25.0	24.8
18:2(5,11)	29.3	27.0	49.6	52.0	47.8	44.3	48.0	49.7
18:cyc(11,12)	5.4	8.0	2.0	2.1	2.0	3.4	1.4	2.0
19:1(11)	0.4	0.6	0.1	0.2	--	0.4	--	0.4
Total	99.9%	99.9%	99.9%	100.0%	99.9%	100.0%	100.1%	99.9%
% Saturated	8.7	8.3	4.9	4.2	6.8	7.4	5.4	7.9
% Monounsaturated	27,8	26.6	26.3	27.8	28.3	28.1	29.6	30.1
% Diunsaturated	40.0	35.1	58,8	59.5	58.4	53.8	57.7	56.1
% Cyclopropane	23.4	29.9	9.9	8.5	6.4	10.7	7.4	5.8

average of two determinations) are similar, but not identical to the differences observed in the membrane fractions isolated from vegetative cells. The most prominent change incurred by growing amoebae at the lower temperature is an overall increase in the level of the endogenous doubly unsaturated fatty acids, with a coincident reduction of the bacterial cyclopropane fatty acids. This observation favors the hypothesis that homeoviscous adaptation plays a role in the regulation of membrane fluidity in Dictyostelium.

The profiles of fatty acids from amoebae grown and starved for 16 hrs at either 18° or 23.5° demonstrate that a very large turnover of cellular lipid occurs during the course of starvation. The bacterial cyclopropane fatty acids decrease by roughly 60-70%, saturated fatty acids decrease by ≃ 50% and the proportion of di-unsaturated fatty acids increase by roughly 50-70%. Curiously, starvation in the absence of development seems to eliminate the differences in the fatty acid composition of vegetative cells grown at either 18° or 23.5°C. The overall level of endogenous unsaturated fatty acids is in fact slightly greater in 23.5° than in 18° grown/starved amoebae.

The selective removal of cyclopropane fatty acids and replacement with endogenous unsaturated fatty acids is evident for pseudoplasmodial as well as starved amoebae. In contrast, however, when normal aggregation and pseudoplasmodium formation are allowed to proceed there is, as in vegetative amoebae, evidence for homeoviscous adaptation when the fatty acid composition of 18° versus 23.5° pseudoplasmodial amoebae are compared. In an average

of three determinations, amoebae from newly formed (16 hr) pseudoplasmodia showed the same increase in diunsaturated fatty acids and concommitant decrease in cyclopropane fatty acids as those observed in vegetative amoebae at the lower temperature of growth/development.

After many hours of migration, amoebae from "lipid-depleted" (34 hr) pseudoplasmodia had a somewhat different fatty acid profile at the two temperatures of growth and development. The difference in the proportion of diunsaturated fatty acids was less pronounced than that in vegetative or 16 hr pseudoplasmodial amoebae. In addition, there was an increase in the level of cyclopropane fatty acids with a coincident decrease in saturated fatty acids at 18°C relative to 23.5°C. One other point of interest was the increase in the ratio of octadecadienoic (18:2) to hexadecadienoic (16:2) acids at the higher temperature. Although the fatty acid profile of amoebae from aged pseudoplasmodia is quite different from that of amoebae from young pseudoplasmodia at the two temperatures of growth and development, the proportions of the various fatty acids still indicate the retention of more fluid lipids at the lower temperature. Some of the changes observed in the fatty acid composition of aged versus newly-formed pseudoplasmodia may be due to the culmination of some of the pseudoplasmodia. After 34 hrs incubation quite a few more sorocarps were formed at 23.5°C than at 18°C, possibly due to drying of the agar plates.[19]

3. Complete characterization of lipids from pseudoplasmodial amoebae

Results from the extraction and fractionation of total lipids from large batches of 18° (23 hr incubation) and 23.5° (20

hr incubation) pseudoplasmodial amoebae are presented in Tables IIIA-IIID. Table IIIA gives the total amount of lipid extracted from each batch of amoebae and the ratio of lipid to lipid-free dry weight. The 18° amoebae yielded 1.6x as much lipid as the 23.5° amoebae, but the actual increase in lipid relative to dry weight was only 1.1x at the lower temperature.

Table IIIB presents the results of fractionation of total lipids by silicic acid column chromatography into the three major classes of neutral lipid, glycolipid and phospholipid. Virtually all of the lipid applied to the columns was recovered for both extracts. There was, in fact, a slight increase in the total mass of material recovered, which was attributable to the inclusion of a few grains of silicic acid in each of the three fractions. The most striking difference in the major lipid fractions from 23.5° compared with 18° pseudoplasmodial amoebae was the proportion of "glycolipid", which accounted for 15.7% of the total lipid from the former and only 1.3% of the total lipid from the latter. However, it is not known whether any runover of phospholipid into the glycolipid fraction occurred, as this fraction was not analyzed for sugar or phosphate content.

Table IIIC gives the percentage of the neutral lipid fractions accounted for by free sterols, and the proportions of stigmastenol and stigmastanol, which were determined by GLC after fractionation of the neutral lipids by TLC. The ratio of free sterol to both total neutral lipid and phospholipid was higher for 18° compared with 23.5° pseudoplasmodial amoebae, and the ratio of

Tables IIIA - IIID. Fractionation and Quantitation of Lipids from Pseudoplasmodial amoebae

Table IIIA

Lipid Extraction	Development 23.5°C 20 hr Pseudoplasmodia	Development 18°C 23 hr Pseudoplasmodia
Total lipid extracted:	23 mg	26.5 mg
Lipid-free dry weight:	190 mg	267.5 mg
mg lipid/g dry weight:	121 mg	137 mg

Table IIIB

Silicic Acid Column Fractions		
Neutral lipid - %:	5.2 mg - 21.5%	9.9 mg - 26.0%
Glycolipid - %:	3.8 mg - 15.7%	0.5 mg - 1.3%
Phospholipid - %:	15.2 mg - 62.8%	27.7 mg - 72.7%
Lipid recovery - %:	24.2 mg - 105% *	38.1 mg - 104% *

Table IIIC

GLC of Neutral Lipid		
mg free sterol:	2.44 mg	5.96 mg
Free sterol % neutral lipid:	46.9%	60.2%
mg stigmastenol - % sterol:	2.28 mg - 93.3%	5.15 mg - 86.5%
mg stigmastanol - % sterol:	0.16 mg - 6.7%	0.81 mg - 13.5%

Table IIID

TLC of Phospholipid	PO_4%	Centimeters from origin	PO_4%	Centimeters from origin
Phosphatidyl inositol:	2.7%	1.0	3.2%	1.0
Lysophospholipids (LPC & LPE):	28.2%	2.4	20.7%	2.3
Phosphatidyl choline:	21.6%	4.6	24.6%	4.0
Phosphatidyl ethanolamine:	47.5%	9.2	47.7%	9.0
Phosphatidic acid:	ND †	--	3.8%	12.5
Solvent front:	--	19.5	--	19.4

*% in excess of 100 due to grains of silicic acid

†May have been present in small % but band too disperse to detect.

the minor sterol stigmastanol to the major sterol stigmastenol was also slightly higher. The recrystallization of steryl acetates prior to GLC analysis may have resulted in the loss of material, and therefore may have introduced some error into these determinations.

Table IIID presents data from the one dimensional thin-layer chromatographic separation of the phospholipid fractions from 18° and 23.5° pseudoplasmodial amoebae. Four or five phospholipid spots were separated and tentatively identified on the basis of co-chromatography with authentic standards and reaction with specific spray reagents. The percentage of total phopholipid phosphorus in each spot was quite similar for the two temperatures of growth and development. A slight increase in the ratio of lysophospholipids (lysophosphatidyl choline and lysophosphatidyl ethanolamine were unresolved) to phosphatidyl choline was observed for 23.5° compared with 18° pseudoplasmodial amoebae. Also, phosphatidic acid, a minor phospholipid in 18° amoebae, was not detected on the chromatogram of phospholipids from 23.5° amoebae. This may, however, have been due to the broad spread of phospholipid bands on the upper-most part of the TLC plate.

Steryl esters separated from the neutral lipid fraction by thin-layer chromatography, as well as glycolipid and phospholipid fractions separated on silicic acid columns, were transesterified with sodium methoxide. Table IV presents a comparison of the fatty acyl methy ester profiles from these three lipid fractions for the two temperatures of growth and development. A large increase in the proportion of diunsaturated fatty acids in all three fractions was

Table IV. Fatty Acid Composition of the Three Major Lipid Fractions from Pseudoplasmodial Amoebae

Fatty acyl methyl ester	NC-4 18°C 23 hr Pseudoplasmodia			NC-4 23.5°C 20 hr Pseudoplasmodia		
	Steryl esters	Phospho-lipids	Glyco-lipids	Steryl esters	Phospho-lipids	Glyco-lipids
14:0	--	--	0.6	0.5	--	0.1
15:0	0.2	0.3	0.2	0.6	0.4	0.2
16:0	5.0	4.6	4.2	5.4	6.0	3.3
16:1(9)	0.5	1.5	6.4	1.5	3.4	15.7
16:2(5,9)	13.5	16.7	7.5	6.2	9.3	4.3
17:0	--	0.7	--	1.3	3.1	1.8
16:cyc(9,10)	14.3	6.8	9.6	19.8	9.4	19.0
17:1(9)	5.2	1.7	--	6.0	1.7	1.0
18:0	--	--	--	0.7	0.1	--
18:1(11)	5.3	20.2	11.9	7.2	23.9	19.8
18:2(5,11)	52.5	46.0	54.5	43.4	37.4	29.8
18:cyc(11,12)	0.9	1.0	1.6	3.4	4.5	3.2
19:1(11)	2.1	0.5	1.9	2.3	0.9	1.5
Others	0.6	--	1.5	1.7	--	0.3
Total	100.1%	100.0%	99.9%	100.0%	100.1%	100.0%
% Saturated	5.2	5.6	5.4	8.5	9.6	6.4
% Monounsaturated	13.1	23.9	20.2	17.0	29.9	36.5
% Diunsaturated	66.0	62.7	62.0	49.6	46.7	34.1
% Cyclopropane	16.4	7.8	11.2	23.2	13.9	22.2

shown for 18° relative to 23.5° pseudoplasmodial amoebae, with a coincident reduction in the levels of cyclopropane, saturated and monounsaturated fatty acids. Particularly striking was the increase in the hexadecadienoic acid, 16:2(5,9), which ranged from ≃ 1.7-2.2x higher in the three fractions from 18° compared with 23.5° amoebae. The lipid fractions in this experiment showed a much larger difference in fatty acid composition for the two temperatures of growth and development than did the total lipid fractions in the previous experiment. One possible explanation for this difference is that the developmental age at which the pseudoplasmodia were harvested in the complete lipid characterization experiment was optimal in terms of differences in fatty acid composition related to the temperature of growth and development. In addition, the 18°C pseudoplasmodia were harvested 3 hrs later (23 hrs) than the 23.5°C pseudoplasmodia (20 hrs), which could account for some of the increase in the diunsaturated fatty acids and concommitant loss of cyclopropane and saturated fatty acids. This "staggering" of the times at which 23.5° and 18° pseudoplasmodia were harvested was introduced into the experiment because the processes of aggregation and development require ≃ 2 additional hours at the lower temperature. Differences in the fatty acid composition of K. aerogenes cultures fed to 18° versus 23.5° amoebae may also have had some influence on the results. The ratio of monoenoic to cyclopropane fatty acids in bacterial lipids fluctuates with changes in the available nutrients and the phase of growth.[52,64] Such differences appear to have little effect on the fatty acid composition of

Dictyostelium amoebae beyond the vegetative stage of growth, as the bacterial fatty acids are selectively catabolized and replaced by the slime mold's endogenous mono- and dienoic fatty acids (however, see section II fatty acid "feeding" experiments).

The fatty acid composition of the phospholipid fraction for the two temperatures of growth and development is most pertinent to the question of homeoviscous adaptation in Dictyostelium. As would be expected, considering the greater proportion of phospholipid relative to other cellular lipids, the fatty acid profiles from this fraction most closely resembled the fatty acid composition of total lipids extracted from 16 hr pseudoplasmodial amoebae. A trend which is evident in the fatty acid composition of phospholipids from 18° (23 hr) compared with 23.5° (20 hr) pseudoplasmodial amoebae is an increase of the dienoic acids with a corresponding decrease in the monoenoic acids for both the 16 and 18 carbon "families". This observation is consistent with a mechanism for homeoviscous adaptation in Dictyostelium involving the low temperature activation of a particular desaturase enzyme which further desaturates 9 or 11-monoenoic fatty acids at the 5 position.[62,98,99]

4. Heavy isotope double label experiment using GC-MS:

From physiological experiments, the last few hours of development were determined to be the most critical time for temperature-induced adaptation of thermosensing in Dictyostelium (see section III). It is probable then that if homeoviscous adaptation is the underlying mechanism of thermal adaptation of thermotaxis,

a temperature jump imposed at the beginning of development would exert its effect by modifying the course of the rapid turnover of lipid which has been shown to occur during that time.[91] The heavy isotope double label experiment was performed to determine how a temperature jump imposed at the start of development affects the fatty acid composition of phospholipids in Dictyostelium. Specifically, the question addressed was whether either de novo synthesis or a partial degradation and restructuring of membrane phospholipids is involved in the incorporation of more highly unsaturated fatty acids into cellular membranes when the temperature of development is lowered.

Carbon-13 or deuterium labelled acetate was used for this experiment because: 1) starvation is the "signal" for the onset of development in Dictyostelium,[100] and the presence of either sugars[101] or amino acids[102] can inhibit aggregation; 2) Dictyostelium can elongate fatty acids by the addition of acetate at the carboxyl end;[99] and 3) sodium acetate at a concentration of 20mM was found to have no adverse effect on aggregation or pseudoplasmodium formation.

The results of the heavy isotope label experiment are presented in Table V. With the technique used for the mass spectrometric analysis (chemical ionization with isobutane) the predominant charged species for each fatty acyl methyl ester is the molecular ion plus one, or M^++1. The unlabelled acetate control provides a measure of the natural abundance of heavy isotopes in the various fatty acids, as indicated by the percentages of the M^++2 and M^++3 species relative to M^++1. If either de novo synthesis

Table V. Mass Spectrometric Distribution of Ions from Fatty Acyl Methyl Esters

Fatty acyl methyl ester	Molecular weight	Unlabelled acetate 19 hr Pseudoplasmodia Development: 23.5°C Abundance of ion			D_3-labelled acetate 20 hr Pseudoplasmodia Development: 23.5°C Abundance of ion			^{13}C-labelled acetate 20 hr Pseudoplasmodia Development: 18°C Abundance of ion		
		M^++1	M^++2	M^++3	M^++1	M^++2	M^++3	M^++1	M^++2	M^++3
16:0	270	100.0	21.8	2.5	100.0	20.7	3.0	100.0	22.6	4.6
16:1(9)	268	100.0	19.0	2.4	100.0	19.4	3.5	100.0	20.1	3.3
16:2(5,9)*	266	100.0	23.4	4.2	100.0	21.4	7.1	100.0	24.7	5.7
17:0*	284	26.2	6.1	--	60.3	15.1	--	32.9	10.8	3.8
16:cyc(9,10)	282	100.0	20.9	6.4	100.0	22.0	7.7	100.0	25.4	4.2
18:1(11)	296	100.0	20.2	2.8	100.0	22.0	3.6	100.0	23.6	3.9
18:2(5,11)	294	100.0	23.5	4.9	100.0	23.6	4.1	100.0	25.5	4.3

*Peaks unresolved on SP-2330 column.

or partial degradation and restructuring of membrane phospholipids were rapidly taking place during development, one would expect the incorporation of two or more labelled acetate moieties into a small fraction of the fatty acids in amoebae incubated with labelled acetate. The mass spectra from fatty acids of pseudoplasmodial amoebae incubated with labelled acetate would then show at least a trace of the M^++4 and M^++5 molecular species. This did not occur for either pseudoplasmodia formed in the presence of 20mM 2-D_3 sodium acetate at 23.5° or pseudoplasmodia formed in the presence of 20mM 1-carbon 13 sodium acetate at 18°. However, an increased percentage of the M^++2 and M^++3 ions of most fatty acids was evident for both the deuterium and carbon-13 labelled pseudoplasmodia. The magnitude of this increase was quite small, leading to the conclusion that very little of the heavy isotope labelled acetate was incorporated into the fatty acids of membrane phospholipids. Furthermore, there was no indication of specific incorporation of labelled acetate into unsaturated fatty acids at the lower temperature of development.

These data tend to rule out the involvement of de novo fatty acid synthesis in the changes of lipid composition in Dictyostelium which occur during development. It seems more likely that the changes are due, at least in part, to a selective catabolism of existing lipid. In support of this hypothesis, Long and Coe[91] report that during aggregation the reserves of free fatty acids and glycerides are utilized as an energy source. Once these are depleted, phospholipid is degraded, and the slime mold amoebae exhibit

a steady decline in mg polar lipid per g lipid-free dry weight throughout development.[91] Selective degradation of lipid cannot, however, account for all of the changes in fatty acid composition which are observed. The action of Δ-9 and Δ-5 desaturase enzymes is required for the conversion of saturated and monoenoic fatty acids to the unusual dienoic fatty acids of Dictyostelium, which increase rapidly during starvation and development.[91,91,99]

The fatty acyl methyl esters from the three phospholipid fractions analyzed in the GC-MS experiment were also quantified by standard gas chromatography, and the data are presented in Table VI. Of particular interest is the comparison of the fatty acid profiles from phospholipids of amoebae which were harvested from the same 23.5° grown cultures and subsequently allowed to develop at either 18° or 23.5°C. As in the previous analysis of the fatty acid composition of phospholipids from pseudoplasmodial amoebae, an increase in the dienoic acids with a corresponding decrease in the monoenoic acids was evident at the lower temperature of development. The results are consistent with the hypothesis that homeoviscous adaptation in developing Dictyostelium amoebae takes place via the low temperature activation of the Δ-5 desaturase.

Table VI. Fatty Acid Composition of Phospholipids from Pseudoplasmodia

Fatty acyl methyl ester	Unlabelled acetate 19 hr Pseudoplasmodia Development: 23.5°C	D_3-labelled acetate 20 hr Pseudoplasmodia Development: 23.5°C	^{13}C-labelled acetate 20 hr Pseudoplasmodia Development: 18°C
15:0	0.8	0.7	0.6
16:0	4.8	6.1	5.5
16:1(9)	8.6	4.8	4.3
16:2(5,9)*	6.8	4.4	6.9
17:0*	4.0	2.7	2.1
16:cyc(9,10)	4.4	3.6	3.3
17:2(5,9)*	0.4	0.5	0.6
18:0*	0.3	0.4	0.2
18:1(11)	27.3	28.7	26.1
18:2(5,11)	42.5	47.9	50.3
18:cyc(11,12)	0.1	0.2	0.1
Total	100.0%	100.0%	100.0%
% Saturated	5.9	7.2	6.3
% Monounsaturated	35.9	33.5	30.4
% Diunsaturated	49.3	52.0	57.2
% Cyclopropane	4.5	3.8	3.4

*Peaks unresolved by gas chromatography; percentages determined by mass spectrometry.

DISCUSSION:

Studies of the effect of growth temperature on fatty acid composition have been performed with a wide variety of microorganisms including bacteria,[56,64] yeasts,[57,103,104] algae[65] and protozoans.[105] The generalization derived from these studies is that microorganisms increase the level of their unsaturated fatty acids when subjected to a lower temperature of growth. This phenomenon is considered to be a simple adaptive response to changing environmental temperature which facilitates survival of the organism. However, closer inspection of individual studies reveals that the relationship between growth temperature and fatty acid unsaturation is seldom straightforward. Other parameters which affect the fatty acid composition, such as the level of certain nutrients,[64,106] culture age[64,103,107] and oxygen tension,[107] are often involved. Oxygen tension, for example, is particularly critical for cultures grown in liquid medium, due to the strong temperature dependence of oxygen solubility and the absolute requirement for molecular oxygen by eukaryotic desaturase enzymes.[108,109]

Clearly, a study of the effect of temperature on the fatty acid composition of Dictyostelium discoideum, NC-4, is considerably complicated by the superposition of other variables. One of these is the requirement for growth on bacteria. The bacterium alone is subject to changes of fatty acid composition induced by temperature and other environmental parameters.[64] This problem could be circumvented by growing amoebae on lipid-extracted, autoclaved bacteria[80] or by substituting one of the axenic mutants of Dictyostelium.[74]

However, either of these alternatives could yield results irrelevant to the question of whether changes of cellular lipids are related to thermal adaptation of thermotaxis in Dictyostelium, because all physiology experiments have been carried out with wild-type amoebae grown on live bacteria.

A second variable in the study of the effect of temperature on the fatty acid composition of pseudoplasmodial amoebae of Dictyostelium is the drastic change in the fatty acid profile which occurs during development.[81] This parameter is further complicated by the fact that the rates of aggregation and pseudoplasmodium formation are temperature dependent.[110] A rapid appearance of the dienoic fatty acids is associated with development in Dictyostelium.[81] Thus, for equal incubation times, a low temperature-induced increase in unsaturated fatty acids during pseudoplasmodium formation might be offset by a more rapid development (and therefore increased dienoic fatty acids) at a higher temperature.

One final consideration is the inordinately high percentage ($\geq$ 90%) of unsaturated fatty acids in the lipids of Dictyostelium. Whereas most organisms specifically acylate the 1-position of phospholipids with saturated fatty acids, Dictyostelium must show no such specificity.[98] A study with fatty acyl chain elongation mutants of Neurospora has shown that there is an absolute requirement for at least a small percentage ($\simeq$ 5%) of saturated fatty acid in membrane phospholipids to support growth.[111] By extrapolation, Dictyostelium must exist on the "brink" of the tolerable limit of fatty acid unsaturation. This probably accounts for the relatively

low temperatures at which optimum growth (≃ 22°C) and cessation of growth (≃ 30°C) occur.[62] Considering the already overwhelming proportion of unsaturated fatty acids in Dictyostelium, any further increase in unsaturation induced by low temperature would undoubtedly be small, particularly in response to a ΔT of only 5.5°C. Furthermore, even a small decrease in the percentage of saturated fatty acids could sharply alter the melting point of membrane phospholipids with such a high proportion of unsaturated fatty acids.[111]

In those microorganisms for which a distinct effect of temperature on fatty acid composition has been demonstrated, one does not observe a fluctuation in the level of all the fatty acids, but rather a change in the ratios of a few select fatty acids. There is also frequently specificity in the class of phospholipid which is modified by a change in temperature. Finally, a linear relationship between temperature and the unsaturation of fatty acids is not always observed. Instead, abrupt changes in the ratios of one group of fatty acids can occur at a certain critical temperature.[108] These features of temperature-induced adaptation of lipids in microorganisms appear to reflect the underlying effects of temperature on the synthesis and activity of specific enzymes involved in phospholipid biosynthesis or restructuring and in fatty acyl desaturation.[108] For example, both of the yeast species Torulopsis utilis and Candida lipolytica show an increase in the ratio of linoleate to oleate at low growth temperature.[57,103] Each organism has separate enzyme systems for the desaturation of either fatty acyl

CoA or fatty acids acylated to phospholipid.[57,58,112,113] The fatty acyl CoA desaturase system alone appears to be responsible for the increased desaturation of oleate to linoleate at low temperature.[57,58] De novo synthesis rather than low temperature activation of the fatty acyl CoA desaturase is the apparent mechanism for increasing the ratio of linoleate to oleate at a lower growth temperature.[57,58] A second example of the effect of temperature on an enzyme involved in phospholipid biosynthesis has been reported for Escherichia coli. Total phospholipids from the bacterium exhibit an increasing ratio of oleate to palmitate with decreasing growth temperature. This temperature-induced adaptation of fatty acid composition was correlated with the low temperature preference for oleate over palmitate as substrate by a particular fatty acyl transferase.[69]

The differences observed in the fatty acid composition of phospholipids from pseudoplasmodial amoebae of D. discoideum, NC-4, which were grown and allowed to develop at either 18° or 23.5°C are consistent with the hypothesis that a single enzyme regulates adaptation. Specifically, the increase in dienoic fatty acids and coincident decrease in monoenoic fatty acids at the lower temperature of growth and development suggest that the Δ-5 desaturase of Dictyostelium is more active at 18° versus 23.5°C. Thermal adaptation of thermotaxis occurs most rapidly during the last several hours of development, when pseudoplasmodia are formed from tight aggregates (see section III). During the same time interval there is a rapid increase in the activity of the Δ-5 desaturase, as evidenced by the rising proportions of the unusual dienoic fatty

acids in lipids of Dictyostelium. Thus, the activity of this enzyme appears to be developmentally controlled. Whether de novo synthesis is involved is not known. In vitro studies of the desaturase activity during the course of development might elucidate this question. In any case, it seems logical that the effect of low temperature would be to further stimulate the process already set into action by the developmental regime.

One peculiarity in the study of the effect of temperature on the fatty acid composition of Dictyostelium which bears further explanation was the absence of any appreciable difference in the level of total dienoic acids in amoebae starved at either 18° or 23.5° while suspended in phosphate buffer. The Δ-5 desaturase is clearly quite active under these conditions, as the levels of the dienoic fatty acids in 16 hr starved amoebae was slightly higher than in 16 hr pseudoplasmodial amoebae. It is possible that a certain degree of control of the desaturase which exists in pseudoplasmodia is not expressed in starved amoebae. In several experiments where cell starvation in phosphate buffer was extended for periods longer than 16 hrs (data not shown) the level of the octadecadienoic acid continued to rise steadily at the expense of all other fatty acids. It should also be noted that an increase in the proportion of hexadecadienoate relative to octadecadienoate was observed for amoebae starved at 18° compared with 23.5°C. Furthermore, the relative distribution of the dienoic fatty acids in phospholipid and steryl ester at the two temperatures of starvation are not known.

The steryl ester fraction from lipids of D. discoideum becomes highly enriched in the dienoic fatty acids during the course of development.[81,91] This observation suggests one additional mechanism whereby the proportion of dienoic fatty acids in phospholipids of pseudoplasmodial amoebae could be elevated at a lower temperature of development. The steryl esters are believed to be a reserve form of fatty acid or sterol, or both.[114] If an enzyme in Dictyostelium which transesterifies fatty acid from steryl ester to phospholipid were either stimulated or synthesized in response to low temperature the observed increase in dienoic fatty acid content of phospholipid would result. A coincident rise in the ratio of free to esterified sterol would also be expected, and this was in fact observed in the neutral lipid fraction from 18° compared with 23.5° pseudoplasmodial amoebae.

SECTION II

MEMBRANE PERTURBATIONS AND THERMOTAXIS IN *DICTYOSTELIUM DISCOIDEUM*

INTRODUCTION:

The two primary lipid constituents of plasma membranes in most simple eukaryotic cells are phospholipids and free sterols. The ratios and interactions of these membrane components determine the temperatures at which, and the sharpness with which, membrane lipid phase transitions occur.[49-51] Phospholipids in a bilayer exhibit co-operative melting from the solid to liquid-crystalline state.[49-51] This accounts for the abruptness of the phase transitions in pure phospholipid membranes.[49,50,71] An increase in the proportion of unsaturated or short chain fatty acids esterified to glycerol in membrane phospholipids lowers the temperature at which a given phase transition occurs.[51,71,72] Free sterols appear to interact with the polar head groups of membrane phospholipids with a set stoichiometry (1:2).[61,115,116] Phospholipids bound with sterol are denied the co-operative interactions exhibited in pure phospholipid bilayers,[59,61,116,117] and thus, sterols have the effect of "spreading" a membrane lipid phase transition out over a much broader temperature range.[50,60,115]

With these fundamentals of the physical chemistry of membrane lipids in mind, two sets of experiments were designed to test the

hypothesis that a membrane lipid phase transition serves as the biothermometer for thermotaxis in Dictyostelium.[35] The first set of experiments involved the utilization of various inhibitors of sterol biosynthesis and function in an effort to abolish or alter the thermotactic response. Conceivably, inhibited synthesis of sterol or precipitation of sterol from the membrane matrix might reduce the breadth of the thermosensory range or eliminate thermotaxis altogether. The second set of experiments entailed several different attempts to alter the fatty acid composition of membrane phospholipids in Dictyostelium discoideum, NC-4, through exogenous fatty acid "feeding". Extensive alteration of cellular lipids in the axenic strain of D. discoideum, Ax_2, has been achieved by supplementing fatty acids sequestered with bovine serum albumin in the growth medium.[82] Unfortunately, strain Ax_2 exhibits diminished thermotactic sensitivity and a reduced rate of migration in comparison with the wild-type strain, NC-4. As NC-4 feeds only on bacteria, it was necessary to supply exogenous fatty acids in some way bound with the bacterial associate. Successful incorporation of more highly unsaturated exogenous fatty acids into membranes of pseudoplasmodial amoebae of NC-4 might have the effect of shifting the thermosensory range for positive thermotaxis to lower temperatures.

MATERIALS AND METHODS:

1. Inhibitors and antibiotics:

Cultures: Dictyostelium discoideum, strain NC-4, was grown in suspension culture in association with Klebsiella aerogenes, harvested and freed of bacteria as described in M & M section I.2.

Inhibitors and thermotaxis: Three inhibitors of steroid biosynthesis (SK&F 7997, Amo 1618 and CCC)[118-120] plus one inhibitor of sterol function (digitonin)[121] were tested for their ability to disrupt normal thermotaxis by pseudoplasmodia of D. discoideum, NC-4. The first step in this procedure was to establish a "threshold" concentration of each compound which produced partial inhibition of aggregation and pseudoplasmodium formation. Washed vegetative amoebae of NC-4 were resuspended in cold distilled water at 10^8 cells/ml and 5µl droplets of the amoebal suspension centered on 2% (w/v) water agar Petri plates. A 5µl droplet containing a given concentration of one inhibitor was then mixed with the amoebal suspension on each plate. The plates were wrapped in foil and incubated in darkness for 24 hrs, then observed for pseudoplasmodium formation in comparison with water controls. Once a "threshold" concentration of each inhibitor was established, thermotaxis was measured on 0.22°C/cm thermal gradients by pseudoplasmodia of NC-4 formed either in the presence or absence of this threshold concentration of inhibitor. Experimental and control 18°C pseudoplasmodia were tested on an 18° → 20°C gradient, whereas experimental and control 23.5°C pseudoplasmodia were tested on a 22° → 24°C gradient.

(See M & M section III for methods of thermotaxis experiments.) The steroid biosynthesis inhibitors Amo 1618 (2-isopropyl-4-dimethylamino-5-methyl phenyl-1-piperidine-carboxylate methyl chloride), CCC [(2-chloroethyl) trimethyl ammonium chloride] and SK&F 7997 [tris-(2-diethylaminoethyl) phosphate trihydrochloride] were obtained from Rainbow Color & Chemical Co., American Cyanamid Co. and Smith, Kline & French Laboratories, respectively. Digitonin was purchased from Sigma Chemical Co.

Amo 1618 and sterol biosynthesis: Biochemical evidence that the steroid biosynthesis inhibitors used in the physiology experiments were exerting their effect on development through a reduction in de novo sterol synthesis was sought. Cultures of D. discoideum, NC-4, grown at either 18° or 23.5°C were washed free of bacteria and divided into two equal portions suspended in phosphate buffer, pH 6.0, at 10^7 cells/ml. A suprathreshold concentration of Amo 1618 (2.5mM - a concentration found to completely inhibit development) was added to one half of the amoebae grown at 18°C and to one half of the amoebae grown at 23.5°C. The experimental and control cultures were then replaced on a rotary shaker at the temperature of growth for 17 hrs (a period of starvation equivalent to that required for pseudoplasmodium formation by amoebae on agar). After the starvation period the cells from each of the four cultures were harvested by centrifugation (750x g, 2 min) and washed in cold distilled water. The total sterol from each cell pellet was recovered after saponification by digitonin precipitation according to the method described by Chance et al.[88] To whole cells suspended in

distilled water was added an equal volume of 15% methanolic KOH. A known quantity of cholesterol was added as an internal standard. The mixture was heated at 70°C for 2 hrs, and the methanol evaporated under a stream of nitrogen. The non-saponifiable fraction was recovered by extracting the aqueous solution 3x with an equal volume of hexane. The hexane extract was dried under a stream of nitrogen, and the residue suspended in 0.5 ml of 95% ethanol. To this suspension 1.0 ml of ethanol, 1.5 ml of acetone and 1.0 ml of 0.5% (w/v) digitonin (Sigma Chemical Co.) in 80% ethanol were added, and the mixture incubated overnight at room temperature. The digitonide precipitate formed was collected by centrifugation, washed once in diethyl ether-acetone, 2:1 (w/v), and twice with diethyl ether, and dried in a vacuum dessicator. Steryl acetates were prepared from the digitonides for GLC quantitation as described in M & M section I.3. Based on the internal cholesterol standard, a comparison was made of the amount of total sterol in cultures with and without Amo 1618 at the two temperatures of growth and starvation.

Polyene antibiotics and thermotaxis: Experiments similar to those performed with inhibitors of sterol biosynthesis were carried out with two polyene fungal antibiotics, amphotericin B and nystatin (mycostatin).[122,123] As these compounds are water-insoluble, it was necessary to add them to aqueous suspensions of D. discoideum, NC-4, amoebae (10^8 cells/ml) at high concentrations in a small volume of dimethyl sulfoxide or dimethyl formamide (1-4µl). Thus, when establishing "threshold" concentrations of the antibiotics, controls containing DMSO or DMF were also observed for inhibition of

development. Threshold concentrations of the antibiotics produced a significant delay in pseudoplasmodium formation. It was therefore necessary to spot droplets of antibiotic-treated amoebae on plates 8-9 hrs before the controls to ensure that experimentals and controls were at the same stage of development when tested for thermotaxis. Amoebae were grown and permitted to form pseudoplasmodia at 23.5°C. Thermotaxis by "treated" and control (DMSO or DMF) pseudoplasmodia was compared on 0.22°C/cm thermal gradients of 19° → 21°C or 20° → 22°C. (See M & M section III for methods of thermotaxis.) The polyene antibiotics amphotericin B and nystatin (mycostatin) were obtained from E.R. Squibb & Sons.

2. Lipid "feeding" experiments:

Fatty acid supplementation: The first three sets of lipid "feeding" experiments entailed the addition of fatty acids in an ether solution to: 1) lipid-extracted (24 hrs ether; 24 hrs methanol), 2) lyophylized, or 3) lyophylized and autoclaved Klebsiella aerogenes. The fatty acid-supplemented cakes of bacteria were then suspended in sterile phosphate buffer, pH 6.0, and inoculated with spores of D. discoideum, NC-4. Those cultures which exhibited good growth were subcultured for a second cycle (≃ 6-7 generations) on fatty acid supplemented bacteria, then harvested. The harvested amoebae were either extracted or allowed to form pseudoplasmodia on 2% (w/v) water agar prior to lipid extraction. Pseudoplasmodia were harvested, disrupted and the pseudoplasmodial amoebae freed of slime sheath as described in M & M section I.2. Lipids were extracted

from vegetative or pseudoplasmodial amoebae and transesterified to yield fatty acyl methyl esters as described in M & M section I.2. The fatty acyl methyl ester profiles of lipid "fed" amoebae were then compared with the profiles of controls of the same developmental age grown in association with unsupplemented, lyophylized bacteria.

Alteration of endogenous bacterial lipids: In a fourth set of lipid "feeding" experiments, the endogenous fatty acids of Klebsiella aerogenes were modified, thereby altering the fatty acid composition of slime mold amoebae fed the bacterium. Cultures of K. aerogenes were grown at low temperature (16°C) on HL-5 broth medium[74] supplemented with 1% (v/v) Tween 80 (Nutritional Biochemicals) and 400μg/ml oleic or linoleic acid (Sigma Chemical Co.). The bacteria were harvested, washed and lyophilized as described in M & M section I.2. The lipids from a small portion of the low temperature/fatty acid-supplemented bacteria were extracted and transesterified by the methods described in M & M section I.2. The fatty acyl methyl ester profiles were then compared with the profiles from control cultures grown at 23.5°C on unsupplemented HL-5 medium.[74] Amoebae of D. discoideum, NC-4 were grown at 18°C in association with the modified bacteria for two passages, then harvested and allowed to form pseudoplasmodia over 20 hrs at 18°C. The amoebae from these pseudoplasmodia were harvested, and their lipids extracted and transesterified, as described in M & M section I.2. The fatty acyl methyl ester profile was then compared with that of control 20 hr, 18°C pseudoplasmodial amoebae

which were grown in association with "standard" (23.5°C/HL-5 grown), lyophilized K. aerogenes. A comparison was also made of thermotaxis by 23.5°C pseudoplasmodia formed from amoebae grown on "modified" versus "standard" bacteria on 0.22°C/cm thermal gradients from 19° → 21°C and 20° → 22°C. (See M & M section III for methods of thermotaxis.)

RESULTS:

1. Effects of inhibitors of sterol biosynthesis and function on thermotaxis:

Inhibitors and development: The experiments testing the effect of varying concentrations of inhibitors of sterol biosynthesis and function on development in Dictyostelium are summarized in Table VII. "Subthreshold" indicates a concentration of a given inhibitor which produced no obvious inhibition of development. A "threshold" level of inhibitor is defined as a concentration which significantly reduced the number of pseudoplasmodia formed, whereas "supra-threshold" refers to an inhibitor concentration which completely suppressed aggregation and pseudoplasmodium formation.

Very high concentrations of the inhibitors of sterol biosynthesis Amo 1618 and CCC were required to prevent development in the "droplet" experiments, where 5µl of inhibitor solution were mixed with 5µl of amoebal suspension on the surface of 2% (w/v) water agar Petri plates. The high solubility and polar nature of these compounds, which could readily diffuse into and bind with the agar substrate, may account for this fact. This suggestion is supported by the demonstration that inclusion of Amo 1618 in the agar substrate resulted in a 40x reduction in the concentration required to competely inhibit development. On the other hand, the sterol biosynthesis inhibitor SK&F 7997 (also a highly soluble, polar compound) was effective at relatively low concentration compared with Amo 1618 and CCC. This may reflect a greater permeability of the cell membrane of Dictyostelium to this inhibitor, or

Table VII. Effect of Inhibitors of Sterol Biosynthesis and Function on Development

Effect on Development	Inhibitors of Sterol Biosynthesis [in agar] Amo-1618	Amo-1618	CCC	SK&F 7997	Inhibitors of Sterol Function Digiton.	Polyene antibiotics Amph. B	Nystatin
Slight or no Inhib. (SUBTHRESHOLD)	0.5mM	25mM	25mM	100µg/ml (pH 7.2)	50µg/ml (0.2%EtOH)	10µM (0.1%DMSO)	40µM (0.4%DMF)
Partial Inhibition (THRESHOLD)	1.5mM	50mM	50mM	200µg/ml (pH 7.2)	100µg/ml (0.4%EtOH)	20µM (0.2%DMSO)	60µM (0.6%DMF)
Complete Inhibition (SUPRATHRESHOLD)	2.5mM	100mM	100mM	300µg/ml (pH 7.2)	150µg/ml (0.6%EtOH)	50µM (0.5%DMSO)	80µM (0.8%DMF)

alternatively may be a function of greater "potency". The inhibitors of sterol function (digitonin and the polyene antibiotics, amphotericin B and nystatin) were all effective in blocking development in the micromolar concentration range. This "efficiency" is probably due to the stoichiometric interaction of these compounds with membrane sterols.

Amo 1618 and sterol biosynethesis: The effects of 2.5mM Amo 1618 on sterol biosynthesis in D. discoideum amoebae during 17 hrs of starvation at either 18° or 23.5°C are presented in Table VIII. The results show no appreciable difference in the total sterol content of amoebae starved in phosphate buffer with or without Amo 1618. The total sterol content of amoebae grown and starved at 18°C was approximately 65% of that present in amoebae grown and starved at 23.5°C for both inhibitor-treated and control cultures. The apparent failure of Amo 1618 to inhibit de novo synthesis of sterols in Dictyostelium has two explanations. The first possibility is that very little sterol synthesis occurs under the conditions of the experiment. Conflicting reports exist in the literature concerning the synthesis of sterol during development in Dictyostelium. Johnson et al observed incorporation of ^{14}C-mevalonic acid into sterol of Dictyostelium only during vegetative growth.[90] In contrast, the more recent report of Long and Coe showed that synthesis of sterol and steryl ester occurs during the differentiation of pseudoplasmodia.[91] In either case, under the condition of starvation in the absence of development, sterol biosynthesis may be suppressed. A second possibility is that the calculation of mg

Table VIII. Effect of Amo-1618 on Sterol Biosynthesis in Starving Amoebae

	Temperature of Growth and Starvation: 18°C		Temperature of Growth and Starvation: 23.5°C	
	Control	2.5mM Amo	Control	2.5mM Amo
mg Stigmastenol	1.45	1.23	2.74	2.24
mg Stigmastanol	0.16	0.15	0.18	0.10
mg Total sterol	1.61	1.38	2.92	2.34
Packed cell vol.	0.9ml	0.7ml	1.0ml	0.8ml
mg Sterol/ml packed cells	1.79	1.97	2.92	2.93

total sterol/ml packed cell volume did not provide an accurate measure of the differences between amoebae starved in the presence or absence of inhibitor. A considerable amount of cell lysis was evident in both the 18° and 23.5°C amoebal suspensions containing Amo 1618, and a smaller volume of packed cells was recovered from these cultures relative to the controls. Also, far more aggultination of cells (a manifestation of the capacity to aggregate) was observed in control versus inhibitor-treated cultures. The increased fragility of cells starved in the presence of Amo 1618 might be due to a decrease in membrane sterol content.[124] Alternatively, the effects of Amo 1618 on membrane stability, aggregation, agglutination and pseudoplasmodium formation in Dictyostelium may be non-specific and unrelated to sterol synthesis.

Qualitative effects of inhibitors on thermotaxis: "Threshold" concentrations of the three inhibitors of sterol biosynthesis Amo 1618, CCC and SK&F 7997, and the inhibitor of sterol function digitonin, were tested for their ability to alter or disrupt thermotaxis by pseudoplasmodia of D. discoideum, NC-4. Qualitative results were obtained through comparison of thermotaxis by inhibitor-treated pseudoplasmodia with thermotaxis by water controls on identical 0.22°C/cm thermal gradients.

Thermotaxis by 18°C pseudoplasmodia formed in the presence of 50mM Amo 1618 or CCC was compared with thermotaxis by water controls on an 18° → 20°C thermal gradient. Development of pseudoplasmodia on the Amo 1618 plates was slightly retarded, and thus the total distance of migration over 32 hrs was reduced, but otherwise

the strongly positive thermotactic response was indistinguishable from that of water controls. The timing of development on CCC plates was unaffected, and the total distance of migration over 32 hrs was ≃ 1.5x that of the water controls, probably due to a noticeable increase in the size of the pseudoplasmodia.[16,17] Positive thermotaxis by CCC-treated pseudoplasmodia was slightly more directed than that of water controls.

Thermotaxis by 18°C pseudoplasmodia formed in the presence of 200μg/ml SK&F 7997 or 100μg/ml digitonin was compared with thermotaxis by water controls on an 18° → 20°C thermal gradient. In addition, thermotaxis by 23.5°C pseudoplasmodia formed in the presence of "threshold" concentrations of SK&F 7997 or digitonin was compared with thermotaxis by water controls on a 22° → 24°C thermal gradient. The timing of development was unaffected by either SK&F or digitonin. Positive thermotaxis on the "optimal" thermal gradients by pseudoplasmodia formed in the presence of SK&F 7997 or digitonin was equivalent to that of water controls for both temperatures of growth and development.

Quantitative effect of amphotericin B on thermotaxis: Thermotaxis by 23.5°C pseudoplasmodia formed in the presence of 20μM amphotericin B was compared with thermotaxis by control pseudoplasmodia formed in the presence of 0.2% DMSO on both 19° → 21°C and 20° → 22°C thermal gradients. The time required for development of migrating pseudoplasmodia was 23 hrs for amphotericin B plates and 15 hrs for control plates. Quantitative analysis of the directness of thermotaxis was carried out as described in M & M section III. The results of

thermotaxis by amphotericin B versus DMSO control pseudoplasmodia are presented in Table IXA. Only slight differences in the directness of a population of pseudoplasmodia formed in the presence or absence of the polyene antibiotic are evident. For thermotaxis on "borderline" thermal gradients (bounding the temperature of transition from positive to negative thermotaxis - see section III) such small differences of directness are well within the limits of standard deviation (see M & M section III). Moreover, the thermotactic response of amphotericin B versus control pseudoplasmodia is of the opposite sense on 19° → 21°C compared with 20° → 22°C thermal gradients (slightly more negative on the former and slightly more positive on the latter). Thus, if the differences in the directness of thermotaxis were significant, they would imply that the effect of the antibiotic is to "sharpen" the transition from positive to negative thermotaxis (see section III) rather than to shift the transition to a higher or lower temperature.

2. Lipid "feeding" experiments:

Fatty acid supplementation: Attempts to alter the fatty acid composition of D. discoideum, NC-4, by supplementing K. aerogenes with exogenous fatty acids in an ether solution were largely unsuccessful. Spores of NC-4 did not germinate well in association with lipid-extracted Klebsiella supsended in phosphate buffer, and cultures attained a titer of only 1-2 x 10^6 cells/ml (in contrast with ≃ 1-1.5 x 10^7 cells/ml for control cultures). Cakes of the lipid-extracted bacteria suspended in phosphate buffer after

Table IXA. Effect of Amphotericin B on Thermotaxis

Thermal Gradient	Directness of Thermotaxis		
	Control plate 0.2% DMSO	Amphotericin B 20μM/plate 1	Amphotericin B 20μM/plate 2
19° → 21°C	(-) 0.195	(-) 0.229	(-) 0.243
20° → 22°C	(+) 0.097	(+) 0.104	(+) 0.233

Table IXB. Effect of Altered Bacterial Fatty Acid Composition on Thermotaxis

Thermal Gradient	Directness of Thermotaxis		
	Bacterial associate: Growth temperature/Fatty acid supplement		
	23.5°C/No addition	16°C/Oleic acid	16°C/Linoleic acid
19° → 21°C	(-) 0.616	(-) 0.662	(-) 0.732
20° → 22°C	(+) 0.111	(-) 0.158	(-) 0.251

supplementation with 100μg/ml oleic acid did not support growth of Dictyostelium amoebae. Davidoff and Korn[80] routinely grew D. discoideum in association with lipid-extracted E. coli. However the conditions of their study differed in that an aggregateless mutant of Dictyostelium was used, and a detergent containing various fatty acids was added to the buffer.[80]

Lyophilized K. aerogenes supplemented with 100μg/ml of oleic or linoleic acid supported growth of D. discoideum, NC-4, almost as well as unsupplemented, lyophilized bacteria. However, the cultures were invariably discolored, and there was little change in the fatty acid composition of vegetative amoebae grown on the fatty acid-supplemented bacteria. Klebsiella remains viable after lyophilizing (the lyophilized bacterium exhibited growth when returned to HL-5 broth medium), and it is probable that the exogenous fatty acids were metabolized by the bacterium. To circumvent this problem cakes of lyophilized bacteria were autoclaved prior to addition of oleic acid in an ether solution. The lyophilized/autoclaved K. aerogenes supplemented with 40μg/ml oleic acid and suspended in phosphate buffer adequately supported growth of Dictyostelium amoebae. In addition, the fatty acid composition of vegetative amoebae of NC-4 was somewhat altered after growth on the oleic acid-supplemented bacteria, with increased proportions of the mono- and diunsaturated fatty acids at the expense of the cyclopropane fatty acids. Unfortunately, higher concentrations of exogenous oleic or linoleic acid (100-200 g/ml) inhibited growth of Dictyostelium on the lyophilized/autoclaved bacteria. It is probable that high

concentrations of free fatty acids perturb the membranes of D. discoideum[63,82] (the rationale for sequestering exogenous fatty acids with bovine serum albumin in the study of fatty acid "feeding" of D. discoideum, Ax_2, by Weeks[82]).

Altered endogenous bacterial lipids and the fatty acid composition of Dictyostelium: A second series of lipid "feeding" experiments was carried out in which the endogenous lipids of K. aerogenes were first altered by supplementation of unsaturated fatty acids in the growth medium coupled with growth at low temperature. Amoebae of D. discoideum, NC-4, were then grown in association with the "modified" bacteria, harvested and allowed to form pseudoplasmodia. The fatty acid profiles from total lipids of the pseudoplasmodial amoebae were compared with the profile from control pseudoplasmodial amoebae grown on standard (growth at 23.5°C on unsupplemented HL-5 medium) K. aerogenes.

Dramatic changes in the fatty acid profile of Klebsiella aerogenes were produced by growth of the bacterium at 16°C in medium supplemented with 1% Tween 80 and 400μg/ml oleic or linoleic acid, as shown in Table X. Supplementation with oleic versus linoleic acid produced different quantitative changes of individual fatty acids in the bacterial lipids, but qualitatively both exogenous fatty acids (coupled with low temperature) resulted in a decrease in the proportion of saturated fatty acids, particularly myristate (14:0), and a large increase in monoenoic fatty acids with a coincident reduction of the cyclopropane fatty acids. In addition, a small amount (≃ 1%) of linoleic acid was incorporated into bacteria grown on medium supplied with this fatty acid.

Table X. Effect of Altered Bacterial Lipids on the Fatty Acid Composition of *Dictyostelium*

Fatty acyl methyl esters	*Klebsiella aerogenes* (Log phase of growth) Fatty Acid Supplement/ Temperature of Growth			*Dictyostelium discoideum* (20 hr Pseudoplasmodia) Bacterial FA Supplement/ Temp. Growth & Development		
	Control 23.5°C	18:1(9) 16°C	18:2(9,12) 16°C	Control 18°C	18:1(9) 18°C	18:2(9,12) 18°C
14:0	13.2	1.8	3.5	--	--	--
15:0	--	--	--	0.3	0.5	0.4
16:0	41.9	38.9	45.5	5.4	5.3	4.8
16:1(9)	0.7	5.0	14.1	4.0	4.9	6.9
16:2(5,9)	--	--	--	13.8	9.0	9.7
17:0	--	--	--	0.9	0.9	0.7
16:cyc(9,10)	25.5	22.1	12.2	12.1	8.9	2.7
18:0	0.1	0.1	0.2	0.1	0.2	0.3
18:1(9* or 11)	5.6	23.1†	19.6	22.1	23.8	30.5
18:2(5,11)	--	--	--	37.4	44.4	43.0
18:2(9,12)*	--	--	1.0*	--	--	0.4*
18:cyc(11,12)	12.9	9.0	3.6	3.9	2.0	0.5
Total	99.9%	100.0%	100.0%	100.0%	99.9%	99.9%
% Saturated	55.2	40.8	49.2	6.7	6.9	6.2
% Monounsaturated	6.3	28.1	33.7	26.1	28.7	37.4
% Diunsaturated	--	--	1.0	51.2	53.4	52.7
% Cyclopropane	38.4	31.4	16.1	16.0	10.9	3.2

*Exogenously supplied fatty acid

†Partially 18:1(9) based on retention time

Also presented in Table X are the fatty acid profiles of 18°C pseudoplasmodial amoebae which were grown on "standard" versus "modified" bacteria. A direct correlation appears to exist between the proportion of cyclopropane fatty acids in the bacterial lipids and in the fatty acid profiles of slime mold amoebae which fed on the different bacteria. In contrast, the relationship between the proportions of monoenoic 16 and 18 carbon fatty acids in bacterial lipids and the proportions of monoenoic and dienoic 16 and 18 carbon fatty acids in lipids from Dictyostelium amoebae is not straightforward. For example, in spite of the 4x increase in 18:1 fatty acids in bacteria grown with supplementary oleic acid, there was only a slight increase in the proportion of 18:1 fatty acid in D. discoideum amoebae grown on these "modified" bacteria. This may be due to the fact that much of the 18:1 in the "modified" bacterium was oleic [18:1(9)] rather than vaccenic [18:1(11)] acid, the latter being the endogenous 18:1 fatty acid in both K. aerogenes and Dictyostelium. Another peculiarity was the lower percentage of hexadecadienoate [16:2(5,9)] in pseudoplasmodial amoebae grown on "modified" versus "standard" bacteria, even though the proportion of palmitoleic acid [16:1(9) - the immediate precursor of 16:2(5,9) in Dictyostelium][99] was much greater in the "modified" Klebsiella.

The net results of the growth of D. discoideum, NC-4, amoebae on fatty acid-supplemented/low temperature grown bacteria were: 1) an increase in the ratio of 18:2(5,11) to 16:2(5,9); 2) a slight increase in the total diunsaturated fatty acids; and 3) an increase in monounsaturated fatty acids at the expense of

cyclopropane fatty acids. By far the most extensive change was the increased ratio of monoenoic to cyclopropane fatty acids in amoebae grown on linoleate-supplemented K. aerogenes, which reflected a similar change in the fatty acid composition of the bacterial lipids.

The cyclopropane fatty acids and their corresponding monoenoic fatty acid precursors appear to be interchangeable in terms of their respective contributions to overall membrane fluidity.[52] It seems likely, then, that in spite of the dramatic shift in the ratio of these two types of fatty acid in pseudoplasmodial amoebae which were grown on 16°C grown/linoleate-supplemented K. aerogenes, there would be little effect on the temperatures at which major membrane lipid phase transitions occur. However, the results of the thermotaxis experiment performed on "borderline" thermal gradients of 19° → 21°C and 20° → 22°C (bounding the crossover temperature from positive to negative thermotaxis - see section III) indicate a slight upward shift in the crossover temperature from positive to negative thermotaxis for 23.5°C pseudoplasmodia formed from amoebae grown on "modified" versus "standard" bacteria. Table IXB shows that on both thermal gradients (19° → 21°C and 20° → 22°C) thermotaxis was most negative for pseudoplasmodia formed from amoebae grown on 16°C grown/linoleate-supplemented bacteria. Thermotaxis by pseudoplasmodia formed from amoebae grown on 16°C grown/oleate-supplemented bacteria was less negative, and control pseudoplasmodia (formed from amoebae grown on 23.5°C grown/unsupplemented bacteria) exhibited the least negative thermotaxis. Although the differences in thermotaxis by pseudoplasmodia formed from amoebae grown on "modified" versus

"standard" bacteria were not large, the fact that they were greater on the 20° → 22°C gradient than on the 19° → 21°C gradient is consistent with the conclusion that these differences are attributable to a "perturbation" of the thermosensor (see section III - thermal adaptation of thermosensing). Furthermore, a direct correlation exists between the extent to which the fatty acid composition of pseudoplasmodial amoebae was altered by growth on the "modified" bacteria (see Table X), and the magnitude of the difference in thermotaxis between "experimental" and control pseudoplasmodia.

DISCUSSION:

The single membrane lipid phase transition-as-biothermometer model proposed by Poff and Skokut[35] to explain positive thermotaxis in Dictyostelium must be re-evaluated in view of the discovery of negative thermotaxis and the adaptive crossover temperature from positive to negative thermotaxis (see section III). It seems unlikely that a membrane lipid phase transition serves as the thermosensor for positive thermotaxis due to the demonstration that the high temperature limit for the response is non-adaptive. However, by the first two-thermosensor model proposed in section III to account for both positive and negative thermotaxis, the possibility remains that an adaptive membrane lipid phase transition establishes the sign of thermotactic migration as positive or negative. If this be the case, then testing the effect of inhibitors of sterol biosynthesis and function on positive thermotaxis in the "optimum" temperature range (i.e. on thermal gradients where maximum positive thermotaxis is observed) may be of limited value. For example, if the effect of a given inhibitor were to: 1) sharpen the transition from positive to negative thermotaxis; or 2) shift the transition from positive to negative thermotaxis to a slightly higher or lower temperature, it is doubtful that any difference would be observed for thermotaxis in the "optimum" temperature range.

Thus, one possible explanation for the failure to observe any effect of the inhibitors of sterol biosynthesis or function (Amo 1618, CCC, SK&F 7997 and digitonin) on thermotaxis was the use of thermal gradients which maximized the response (18° → 20°C for

18°C pseudoplasmodia and 22° → 24°C for 23.5°C pseudoplasmodia). However, the effect of the polyene antibiotic amphotericin B on thermotaxis was tested on "borderline" thermal gradients (bounding the temperature of transition from positive to negative thermotaxis - see section III) with similar negative results. Interpretation of results of thermotaxis on borderline thermal gradients is also clouded by the decreased accuracy of the method used for calculating "directness" in this region of the temperature response curve (see M & M section III).

A second interpretation of the results of the inhibitor study is that, under the condition where pseudoplasmodium formation is completed, sufficient repair of the membrane has occurred to enable "normal" thermotaxis. In the "droplet" experiments testing the effect on thermotaxis of the polar, water soluble sterol biosynthesis inhibitors Amo 1618, CCC and SK&F 7997, the concentration of the inhibitors undoubtedly decreased steadily throughout development via diffusion into the agar substrate. Thus, by the time of pseudoplasmodium formation (when a rise in the levels of sterols and steryl esters is observed)[91] the concentration of these inhibitors may have fallen to a sub-inhibitory level. In addition, these compounds may have caused some non-specific inhibition of development in the experiments where a threshold concentration of each inhibitor was established. In this respect it should be noted that SK&F 525 and SK&F 3301 have reportedly produced non-specific, adverse effects in some fungi[125] and in higher plants,[126] but SK&F 7997 was found to be "mild" by comparison.

Once bound to membrane sterols, it is doubtful that either digitonin or the lipid-soluble polyene antibiotic amphotericin B would be readily displaced. However, new sterol synthesis may have occurred to "repair" the membrane during pseudoplasmodium formation. Damage to fungal membranes produced by digitonin and other saponins can reportedly be repaired in ≃ 2 hrs.[121] Similarly, the ≃ 8 hr delay in development produced by "threshold" concentrations of amphotericin B or nystatin may represent the time required for new sterol synthesis and membrane repair. Notably, sterol biosynthesis in some organisms is subject to "feedback" regulation.[127] Thus, if the amount of free sterol dropped below a critical level, the synthesis of new sterol would be initiated.

In contrast with the majority of the thermotaxis experiments in the inhibitor study, thermotaxis experiments in the lipid "feeding" study were carried out on "borderline" thermal gradients (bounding the crossover temperature from positive to negative thermotaxis - see section III). The temperature of transition from positive to negative thermotaxis, rather than the maximum of positive thermotaxis, was found to be thermally adaptive (section III). Thus, "borderline" thermal gradients provide the most sensitive measure of thermal adaptation of thermosensing in Dictyostelium (see Figure 4). By extrapolation (assuming that thermal adaptation of thermotaxis is brought about by a change in membrane lipids), an effect on thermotaxis produced by lipid "feeding" should also be most readily observed on "borderline" thermal gradients. Thermotaxis by 23.5°C pseudoplasmodia formed from amoebae grown on "modified"

bacteria was more negative on both 19° → 21°C and 20° → 22°C thermal gradients than that of 23.5°C pseudoplasmodia formed from amoebae grown on "standard" bacteria. Moreover, the magnitude of the difference in thermotaxis between lipid "fed" and control pseudoplasmodia was correlated with the increase in the ratio of monoenoic to cyclopropane fatty acids in total lipids of pseudoplasmodial amoebae.

The change in pseudoplasmodial thermotaxis produced by growth of amoebae on bacteria of altered fatty acid composition was small. This is not surprising in view of the equivalent contribution of monoenoic and cyclopropane fatty acids to general membrane lipid fluidity.[52] The initial goal of the lipid "feeding" study -- to introduce more polyunsaturated fatty acids into lipids of pseudoplasmodial amoebae of D. discoideum, NC-4 -- was never realized. Instead, the greatest change in fatty acid composition produced in both Klebsiella and Dictyostelium was a considerable exchange of monoenoic for cyclopropane fatty acids. The study by Weeks on the effect of polyunsaturated fatty acids on the growth and development of D. discoideum, Ax_2, indicated that the differentiation of pseudoplasmodia is sensitive to the fatty acid composition of cell membranes.[82] Moderate incorporation of polyunsaturated fatty acids into cellular lipids resulted in a delay of development which was correlated with membrane repair through selective removal of the polyunsaturated fatty acids.[82] Thus, due to the inhibitory effect of polyunsaturated fatty acids on pseudoplasmodium formation, greater alteration of the thermosensory range of thermotaxis through the incorporation of polyunsaturated fatty acids into lipids of D. discoideum, NC-4, amoebae may not be possible.

One final consideration concerning the possible role of a membrane lipid phase transition as a thermosensor for thermotaxis in Dictyostelium is the question of which cellular membrane is involved. The membrane perturbation studies described above were all based on the assumption that the plasma membrane is the controlling element in the thermoresponses of D. discoideum pseudoplasmodia. The possibility remains, however, that some other cellular membrane could be the site of thermosensing. For example, lipid phase transitions of mitochondrial membranes appear to play a critical role in the chilling sensitivity of vascular plants.[67,68] There is evidence that the photoreceptor pigment for phototaxis by pseudoplasmodia of D. discoideum is localized on the mitochondrion[37,38] (although this has not been demonstrated unequivocally[48]). Thus, it seems reasonable to assume that mitochondria could play a role in thermotaxis as well. If so, the low ratio of sterol to phospholipid in mitochondrial membranes of fungi[125] is one explanation for the ineffectuality of inhibitors of sterol biosynthesis and function on thermotaxis in Dictyostelium. Also, phase transitions of mitochondrial membrane lipids could be sharper and more adaptive than those of plasma membrane lipids, depending on the phospholipid composition.[50,71] Electron paramagnetic resonance spectroscopy studies of D. discoideum plasma membranes have shown three major phase transitions at roughly 15°, 19° and 30°C, none of which appear to change significantly with development or with a change in growth temperature.[128-130] These data do not exclude the possibility that a phase transition by a minor lipid constituent of the plasma

membrane serves as a thermosensor in Dictyostelium. Alternatively, EPR studies with mitochondria or other cellular membranes from Dictyostelium amoebae may reveal an adaptive membrane lipid phase transition which could be correlated with the adaptation of the crossover temperature from positive to negative thermotaxis.

SECTION III

THERMAL ADAPTATION OF THERMOSENSING AND NEGATIVE THERMOTAXIS IN *DICTYOSTELIUM DISCOIDEUM*

INTRODUCTION:

The temperature dependence of positive thermotaxis by pseudoplasmodia of *D. discoideum*, NC-4, was measured by Poff and Skokut[35] as a first step toward a characterization of the thermosensor or biothermometer which controls thermotaxis. On 0.11°C/cm thermal gradients, positive thermotaxis was observed over the narrow range from 21.5° to 28.5°C, with a maximum response at the temperature of growth and development, 23.5°C. Growth (but not development) of amoebae at a lower temperature (20°C) resulted in a slight shift of the low temperature limit for positive thermotaxis. Based on these criteria -- that the thermosensory range was narrow, centered about the temperature of growth and development, and thermally adaptable, Poff and Skokut proposed that a membrane lipid phase transition might serve as the biothermometer for thermotaxis in *Dictyostelium*.[35]

The first part of this study was intended as an expansion of the work of Poff and Skokut on thermal adaptation of thermosensing in *Dictyostelium*. Specifically, the question addressed was whether growth and development of the organism at a temperature well below 23.5°C would result in a complete shift of the

thermosensory range for positive thermotaxis, i.e. such that the thermosensory range would be centered about the new temperature of growth and development. A result of this initial study was the observation of negative thermotaxis, which was then also characterized for the two temperatures of growth and development, 23.5° and 18°C. When it became evident that the crossover temperature from positive to negative thermotaxis, rather than the peak of positive thermotaxis, was thermally adaptable, the crossover temperatures for two additional temperatures of growth and development (16° and 27.5°) were established. Finally, dose response data on the thermal sensitivity of Dictyostelium thermotaxis (Poff and Skokut)[35] indicate that the thermotactic response was nearly "saturated" on the 0.22°C/cm gradients employed for the characterization of positive and negative thermotaxis in this study. Therefore, the thermosensory range for positive and negative thermotaxis by 18° and 23.5°C pseudoplasmodia was characterized on shallower (0.11°C/cm), "non-saturating" gradients for the sake of comparison.

The series of experiments described above indicated that the temperature during development is clearly important in determining the subsequent thermosensory range of the pseudoplasmodia. Therefore, a second part of the study of thermal adaptation of thermosensing in Dictyostelium was undertaken to: 1) measure the rate at which thermal adaptation of the thermotactic range occurs, and 2) establish the time during development, if any, at which a temperature jump is most effective in producing thermal adaptation of subsequent thermosensing. Three sets of temperature jump

(T-jump) experiments were included in this second part of the study. In the first set of experiments amoebae grown at one temperature (T_1) were transferred to a second temperature (T_2) for the entire $\simeq$ 16 hrs of development. For the second set of T-jump experiments the period of development was divided into two incubation periods of varying length, one at T_1 and one at T_2, where T_1 = the temperature of growth. The third set of T-jump experiments was conducted to obtain kinetics curves for thermal adaptation of thermotaxis during the final hours of development. In these experiments, amoebae grown and allowed to begin aggregation at 23.5° were transferred to 18° for the last 2-15 hrs of aggregation/pseudoplasmodium formation.

MATERIALS AND METHODS:

Cultures: Amoebae of Dictyostelium discoideum, strain NC-4, were grown in liquid shake culture in association with Klebsiella aerogenes, harvested by centrifugation and freed of bacteria as described in M & M section I.2. The washed pellet of vegetative amoebae was resuspended in cold distilled water at 10^8 cells/ml. A 10µl droplet of the amoebal suspension was deposited in the center of each 9 cm plastic Petri plate (Falcon) containing sterile, non-nutrient water agar (2% w/v). The plates were wrapped in foil and migrating pseudoplasmodia were permitted to develop over 14-17 hrs in darkness (the timing of development varies somewhat with incubation temperature). Alternatively, in some experiments, the harvested amoebae were washed and suspended in potassium phosphate buffer at 10^7 cells/ml for one incubation period and then collected, suspended in distilled water at 10^8 cells/ml and deposited onto water-agar plates as above for the second incubation period.

Temperature gradients: Temperature gradients were established across Petri plates held in circular recesses in an aluminum block. Water from two controlled (± 0.05°C over 24 hrs) constant temperature circulating water baths was pumped through conduits on either side of the block, establishing a linear temperature gradient across the block.[35] A thin film of water between the plates and the wells in the block surface ensured even heat transfer. Control plates were incubated under isothermal conditions. Once placed on the temperature gradient the pseudoplasmodia on the agar surface of each plate were permitted to migrate in darkness for 24-32 hrs. Results of

thermotactic migration by a population of pseudoplasmodia were recorded by "shadowgraphing" -- using a Petri plate in place of the negative in an enlarger, an image of the pseudoplasmodia and slime trails on the agar surface was printed directly onto photographic paper.[42]

Temperatures were measured using "mercury-in-glass" thermometers, or thermistor thermometers (Yellow Springs Inst. Co.) which were calibrated against a standard thermometer (precision within $\pm$ 0.05°C), the calibration of which is traceable to the National Bureau of Standards (USA).

Quantitation of thermotaxis: Quantitation of thermotactic migration by the population of pseudoplasmodia on an individual Petri plate was carried out by an alternative method to that used by Poff and Skokut.[35] The more tedious but mathematically more rigorous calculation of the "directness" of thermotaxis used in this study was based on the method of Batschelet for deriving the "dispersion" of a population about the mean direction of migration.[131] The angle to the final position of each pseudoplasmodium on a given shadowgraph was measured on a polar axis centered at the inoculum drop. From these angles, directness was calculated according to the Batschelet equation:

$$\text{Dispersion (directness)} = \gamma = (x^2 + y^2)^{\frac{1}{2}}$$

$$\text{where} \quad x = 1/n \sum_{i=1}^{n} \cos \alpha$$

$$\text{and} \quad y = 1/n \sum_{i=1}^{n} \sin \alpha$$

with n = sample number, and α = the angle measured for each pseudoplasmodium.

Batschelet's term γ is referred to here as "directness" rather than "dispersion" since γ is inversely related to what one generally thinks of as dispersion ($\gamma = 0$ for a randomly oriented population; $\gamma = 1$ for a uniformly directed population). Directness (γ) provides a quantitative measure of the adherence of a population, at a given point in time, to a straight (perfectly direct) path. The calculation of γ for shadowgraphs with a large number of pseudoplasmodia was considerably simplified by the use of a TI59 (Texas Instruments) programmable calculator. A program was written enabling the operator to input the angle to the final position of each pseudoplasmodium and obtain the values x,y and γ (directness) for the entire population.

It should be stressed that the Batschelet method for calculating the directness of thermotaxis considers only the final position of each pseudoplasmodium, and ignores migration rate. As a result of these features, there are a few cases in which "directness" (γ) provides a relatively less accurate index of thermotactic migration and exhibits a broader range of standard deviation for a given number of experiments. The most significant case, in terms of the results of this study, entails the condition where adaptation of thermosensing occurs during the course of an experiment. Under this circumstance, the pseudoplasmodia begin migration in one direction, turn, and complete migration in roughly the opposite direction. The directness of thermotactic migration in such an experiment is determined to be approximately random ($\gamma = 0$), although most of the pseudoplasmodia are oriented in the same direction by the end of the experiment.

RESULTS:

1. The thermosensory range for positive and negative thermotaxis:

Amoebae were grown and permitted to develop at four incubation temperatures ranging from 16° to 27.5°C. Pseudoplasmodia from each temperature of growth and development were centered on 0.22°C/cm temperature gradients with midpoints ranging from 14° to 29°C. The results (Figure 2) show that pseudoplasmodia migrate toward the cooler side of temperature gradients (negative thermotaxis) with a midpoint temperature several degrees below the temperature of growth/development. It is clear from a comparison of the complete thermosensory range for thermotaxis by 18° versus 23.5° pseudoplasmodia (middle two curves) that the high temperature limit for positive thermotaxis is not shifted down significantly by lowering the temperature of growth and development. By contrast, the temperature of transition from positive to negative thermotaxis was shifted down for 18° compared with 23.5° pseudoplasmodia.

Thermal adaptation of the "zero-transition temperature" was further substantiated by characterization of the range of transition from positive to negative thermotaxis for 16° and 27.5° pseudoplasmodia (curves at extreme left and right). For all four temperatures of growth and development, the transition from positive to negative thermotaxis was abrupt, and occurred at ≃ 14.5°C for 16°C pseudoplasmodia, ≃ 17°C for 18°C pseudoplasmodia, ≃ 20.5°C for 23.5°C pseudoplasmodia and ≃ 24°C for 27.5°C pseudoplasmodia.

Figure 3 presents a comparison of the complete thermosensory range for thermotaxis by 23.5°C pseudoplasmodia on 0.11°C/cm versus

Figure 2. Temperature dependence of thermotaxis on 0.22°C/cm gradients for 16°(▲), 18°(●), 23.5°(○), and 27.5°(△) pseudoplasmodia. Vertical bars represent ± 1 standard deviation. The temperature of transition from positive to negative thermotaxis is indicated for each curve to the nearest 0.5°C.

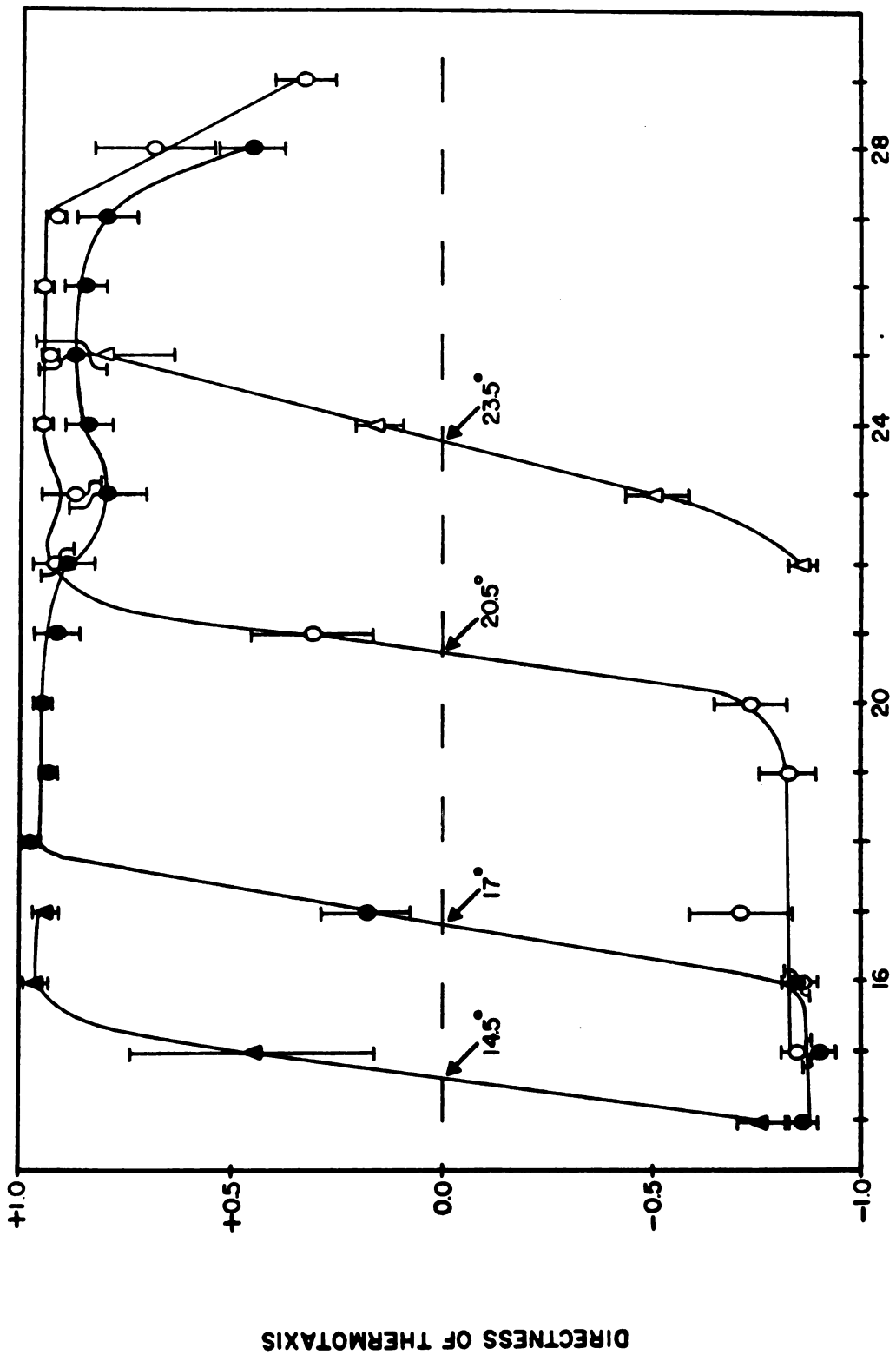
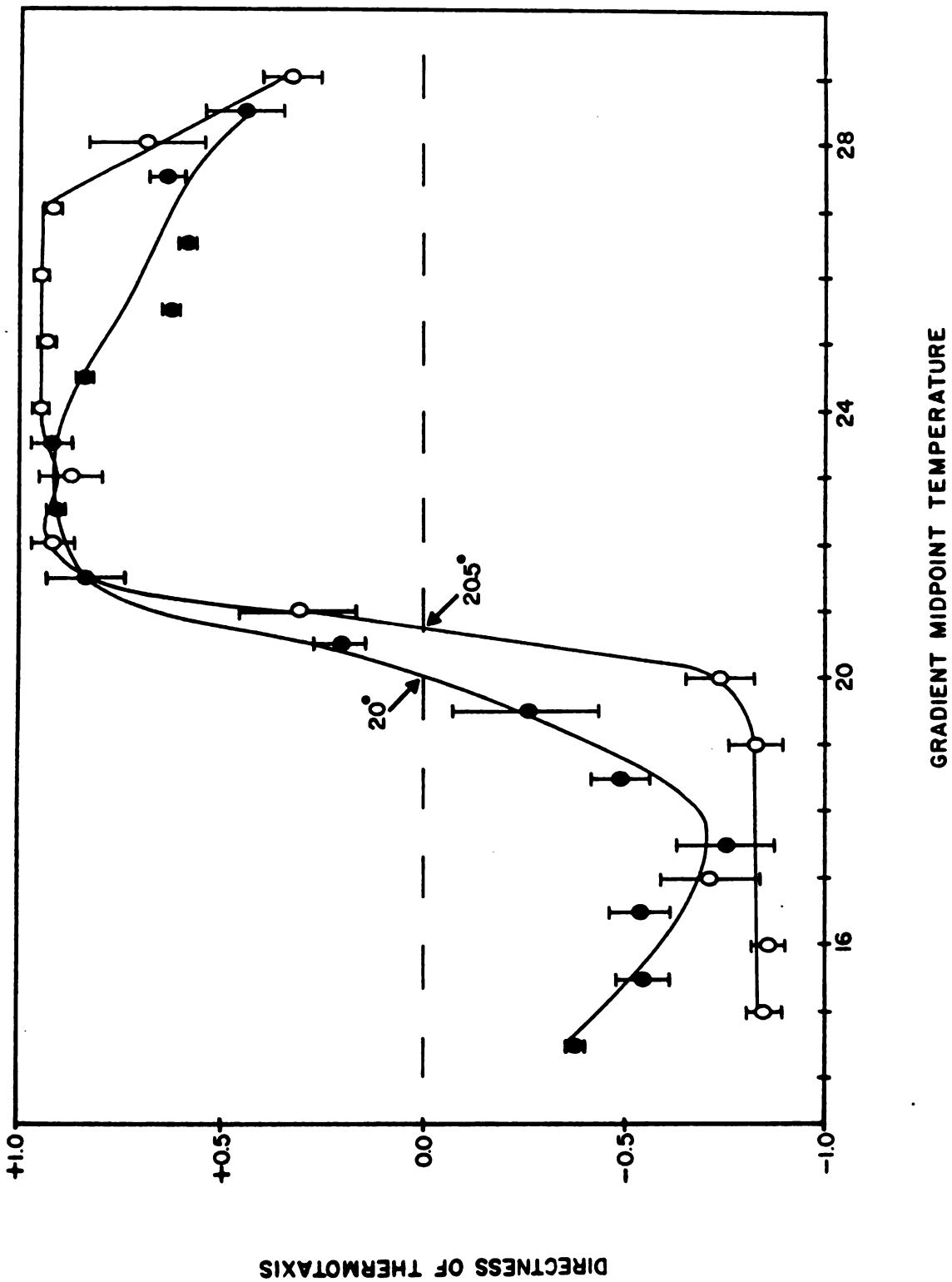

+1.0
+0.5
0.0
-0.5
-1.0
16
20
24
28
14.5°
17°
20.5°
23.5°
DIRECTNESS OF THERMOTAXIS
GRADIENT MIDPOINT TEMPERATURE

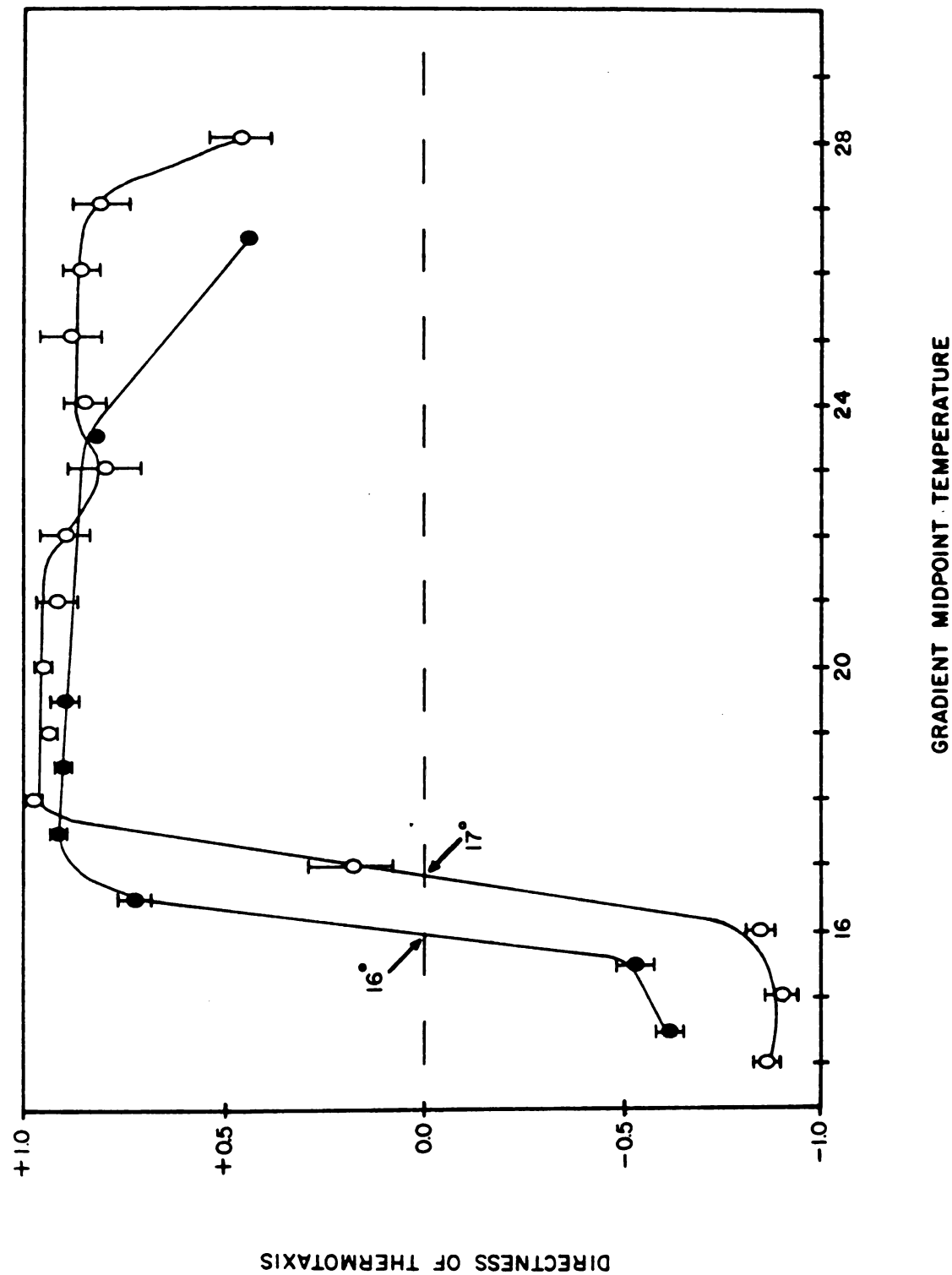
Figure 3. Temperature dependence of thermotaxis on 0.11°C/cm (●), and 0.22°C/cm (○) gradients for 23.5° pseudoplasmodia. Vertical bars represent ± 1 standard deviation. The temperature of transition from positive to negative thermotaxis is indicated for each curve to the nearest 0.5°C.


+1.0
+0.5
0.0
-0.5
-1.0
16
20
24
28
20°
20.5°
DIRECTNESS OF THERMOTAXIS
GRADIENT MIDPOINT TEMPERATURE

0.22°C/cm thermal gradients. The slope of the transition from positive to negative thermotaxis is considerably steeper for pseudoplasmodia migrating on 0.22°C/cm gradients than for pseudoplasmodia migrating on 0.11°C/cm gradients. In addition, the zero-transition temperature from positive to negative thermotaxis is shifted down to a slightly lower temperature for pseudoplasmodia migrating on 0.11°C/cm versus 0.22°C/cm thermal gradients. Thermal sensitivity is decreased (thermotaxis approaches zero) at low temperature ($< \simeq 17°C$) on 0.11°C/cm gradients but not on 0.22°C/cm gradients. At high temperatures ($> \simeq 25°C$) thermal sensitivity drops off more rapidly on 0.11°C/cm than on 0.22°C/cm gradients. These data agree with the report of Poff and Skokut[35] that thermotaxis is nearly "saturated" on a 0.22°C/cm gradient but at approximately 75% of saturation on a 0.11°C/cm gradient.

Figure 4 presents a comparison of the thermosensory range for thermotaxis by 18° pseudoplasmodia on 0.11°C/cm versus 0.22°C/cm thermal gradients. In contrast with the data for 23.5° pseudoplasmodia (Figure 3), the slope of the transition from positive to negative thermotaxis for 18° pseudoplasmodia migrating on either 0.11°C/cm or 0.22°C/cm gradients is approximately the same. There is, however, a similar lowering of the zero-transition temperature for 18° pseudoplasmodia migrating on 0.11°C/cm versus 0.22°C/cm thermal gradients. There is also evidence of reduced thermal sensitivity on 0.11°C/cm relative to 0.22°C/cm gradients at high ($> \simeq 24°C$) and low ($< \simeq 16°C$) temperature.

Figure 4. Temperature dependence of thermotaxis on 0.11°C/cm (●), and 0.22°C/cm (○) gradients for 18° pseudoplasmodia. Vertical bars represent ± 1 standard deviation. The temperature of transition from positive to negative thermotaxis is indicated for each curve to the nearest 0.5°C.


+1.0
+0.5
0.0
-0.5
-1.0
16
20
24
28
16°
17°
DIRECTNESS OF THERMOTAXIS
GRADIENT MIDPOINT TEMPERATURE

2. Thermal adaptation kinetics - temperature jump experiments:

Results from the series of temperature jump experiments in which amoebae were transferred to a new temperature at 0 hrs starvation are presented in Table XI. The data show that significant adaptation occurs in the ≃ 16 hrs required for aggregation and pseudoplasmodium formation. Pseudoplasmodia formed at 16°C from amoebae grown at 16°C are strongly positively thermotactic on a 0.22°C/cm gradient centered at 17°C, whereas pseudoplasmodia formed at 23.5°C from the same amoebae (grown at 16°C) are strongly negatively thermotactic on this gradient. However, "complete" adaptation does not occur during the 16 hrs of development at a new temperature; i.e. the pseudoplasmodia do not behave as though they have been exposed to T_2 throughout cell growth and development. The thermal adaptation of thermosensing is most readily observed on thermal gradients bordering the temperature of transition from positive to negative thermotaxis. For example, pseudoplasmodia were formed at either 16°, 18°, or 23.5°C from cells grown at 18°C, and centered on a 0.22°C/cm gradient from 16° → 18°C. The pseudoplasmodia formed at 18°C exhibited close to random migration on this gradient, whereas those formed at 16°C were strongly positively thermotactic and those formed at 23.5°C were strongly negatively thermotactic.

Varying the time during development at which T_2 was presented showed (Table XII) that a temperature jump from 23.5° to 18°C is as effective in inducing the thermal adaptation of thermotaxis when given at 4 hrs as when given at 0 hrs starvation.

Table XI. Dependence of Thermotaxis on Temperature: Temperature Jump Between Growth and Development

Thermal Gradient[1] (°C)	Growth Temp. (°C)	Incubation Temp. (°C)	Directness
16 - 18	23.5	16	-.62
		18	-.66
		23.5	-.7
		27.5	-.30
18 - 20	23.5	18	+.53
		23.5	-.83
		27.5	-.76
16 - 18	18	16	+.92
		18	+.18
		23.5	-.89
18 - 20	18	18	+.94
		23.5	+.05
		27.5	-.16
16 - 18	16	16	+.95
		23.5	-.89

[1]measured across a 9 cm Petri plate.

Table XII. Dependence of Thermotaxis on Temperature: Temperature Jump During Development

Thermal Gradient[1] (°C)	Growth Temp. (°C)	T_1 (°C)	T_2 (°C)	Time of Jump[2]	Directness
18 - 20[3]	23.5	18	18	-	+.76
		23.5	18	4	+.75
		18	23.5	11	-.56
		23.5	23.5	-	-.82
	18	18	18	-	+.85
		18	23.5	3	-.03
		18	27.5	3	-.33
19 - 21[4]	23.5	23.5	23.5	-	-.84
		23.5	18	8	+.65
20 - 22[4]	23.5	23.5	23.5	-	+.40
		23.5	18	8	+.90

[1] measured across a 9 cm Petri plate.

[2] time (hrs) after cell transfer to starvation conditions (initiation of development).

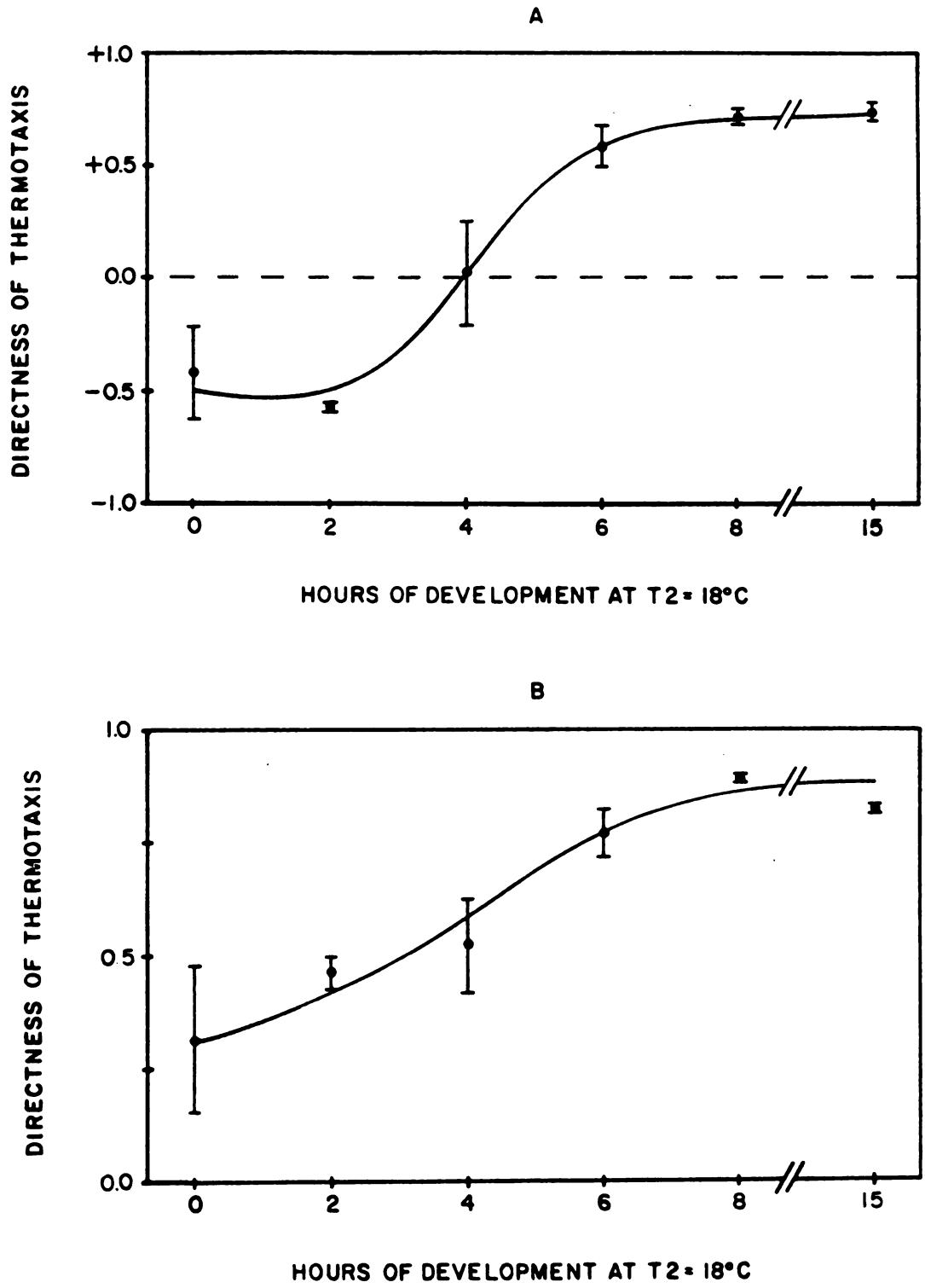
[3] cells in suspension culture during T_1 and on Petri plates during T_2.

[4] cells on Petri plates during T_1 and T_2.

Pseudoplasmodia formed at the growth temperature (23.5°C) for 5 hrs, following 11 hrs of starvation at 18°C, behave thermotactically as though they were incubated at 23.5°C throughout development. In addition, pseudoplasmodia formed over 5 hrs at 18°C, following growth and 11 hrs of starvation at 23.5°C, behave thermotactically almost as though they were incubated at 18°C throughout development. Thus, the final few hours of development, when pseudoplasmodia are formed from tight aggregates, is the most critical time for thermal adaptation of subsequent thermosensing by pseudoplasmodia.

This critical time for thermal adaptation of thermosensing at the end of development was more precisely delimited in the last set of temperature jump experiments. Amoebae grown and incubated at 23.5°C from 0 hrs starvation were transferred to 18°C for the last 2, 4, 6, 8 or 15 hrs of development. Thermotaxis on two "borderline" (bounding the temperature of transition from positive to negative thermotaxis by 23.5°C pseudoplasmodia) 0.22°C/cm thermal gradients (19° → 21°C and 20° → 22°C) was compared with that of control pseudoplasmodia incubated at 23.5°C throughout development. The data are plotted as two curves presenting the kinetics of thermal adaptation during development (Figures 5A and 5B). On the 19° → 21°C gradient (Figure 5A) the directness of thermotaxis varied from ≃ (-)0.50 for 23.5°C pseudoplasmodia (controls) to ≃ (+)0.80 for pseudoplasmodia formed from amoebae which were grown at 23.5°C, then incubated at 18°C throughout development (15 hrs). No change in directness of thermotaxis relative to 23.5°C controls was observed when plates spotted with 23.5°C grown amoebae were incubated at the

Figures 5A and 5B. Curves of the kinetics of thermal adaptation of thermotaxis produced by a jump to 18°C for successively longer time intervals at the end of development, after growth and partial development of amoebae at 23.5°C.
(A) Directness of thermotaxis on a 19° → 21°C gradient;
(B) Directness of thermotaxis on a 20° → 22°C gradient.
Vertical bars represent ± 1 standard deviation.


A
+1.0
+0.5
0.0
-0.5
-1.0
DIRECTNESS OF THERMOTAXIS
0
2
4
6
8
15
HOURS OF DEVELOPMENT AT T2 = 18°C
B
1.0
0.5
0.0
DIRECTNESS OF THERMOTAXIS
0
2
4
6
8
15
HOURS OF DEVELOPMENT AT T2 = 18°C

temperature of growth for 13 hrs, then transferred to 18°C for the final 2 hrs of development. In contrast, when incubation was allowed to proceed at the temperature of growth (23.5°C) for 11 hrs, and plates were then transferred to 18°C for the last 4 hrs of development, a positive shift in the directness of thermotaxis was clearly evident. A further positive shift of directness was observed on plates incubated at 18°C for the final 6 hrs of development, and for plates incubated at 18°C for the last 8 hrs, thermotaxis was equivalent to that on plates incubated at the lower temperature throughout development (15 hrs). Thus, the highest rate of thermal adaptation of subsequent thermosensing occurs between the last 2-6 hrs of development, as indicated by the steepness of the slope of the thermal adaptation kinetics curve during this time interval. In addition, thermal adaptation of thermosensing is "complete" after incubation at 18°C for the final 8 hrs of development (i.e. no further positive shift of directness occurs with increasing time of development at 18°C).

Similar results were obtained on the 20° → 22°C thermal gradient (Figure 5B). The directness of thermotaxis varied from ≃ (+)0.30 for 23.5°C pseudoplasmodia (controls) to ≃ (+)0.85 for pseudoplasmodia formed from amoebae which were grown at 23.5°C, then incubated at 18°C throughout development. Some slight adaptation was evident on plates transferred to 18°C during the final 2 hrs of development, but the highest rate of adaptation occurred between the last 2-6 hrs of development. In addition, the extent of the positive shift of directness following incubation at 18°C for the

final 8 hrs of development equalled that following incubation at 18°C throughout development (15 hrs).

DISCUSSION:

The observation of negative thermotaxis by pseudoplasmodia of D. discoideum considerably complicates the simple one thermosensor mechanism which has been suggested to explain their thermotaxis. Poff and Skokut proposed that a membrane lipid phase transition might serve as the "biothermometer" in D. discoideum.[35] This proposal, based on the narrow temperature range for positive thermotaxis, and the temperature-dependent adaptation of that range, must now be modified to account for negative thermotaxis.

Two major arguments which can now be raised against the proposal that a phase transition functions as the biothermometer for thermotaxis are: 1) the high temperature maximum for thermotaxis seems to be non-adaptive (although the transition temperature between positive and negative thermotaxis is adaptable); and 2) the range of the thermotactic response (> 14°C including both positive and negative thermotaxis) is probably too broad to be based on a single transition.

The complexity of the temperature response curves (Figure 2) is difficult to reconcile with a single, temperature-dependent process or biothermometer. However, both positive and negative thermotaxis, and the adaptive temperature of transition from one to the other, could be explained given two such processes. For example, one temperature-dependent process or transition could be the biothermometer for positive thermotaxis and a second process the biothermometer for negative thermotaxis. The transition from negative to positive thermotaxis would result from competition at that

temperature between the positive biothermometer and the negative biothermometer. Consistent with this hypothesis are the data from Figures 3 and 4 which show a different temperature of transition between positive and negative thermotaxis on 0.11°C/cm compared with 0.22°C/cm thermal gradients. These data might be attributable to different temperature dependences for positive and negative thermotaxis.

Alternatively, one temperature-dependent process could serve to measure a temperature differential over the entire range of thermotaxis, while a second process would switch the sign of the thermotactic response from positive to negative. By this hypothesis, the second process would abruptly change with temperature at the transition between positive and negative thermotaxis. A membrane lipid phase transition could function in this capacity, and one might seek a membrane lipid phase change which can be related to the transition temperature at 20.5°C for 23.5°C pseudoplasmodia. Such a phase change would exhibit thermal adaptation dependent upon the growth/developmental temperature of the cells/pseudoplasmodia.

In addition to the two temperature sensors which measure thermal gradients and regulate the sign of thermotaxis in Dictyostelium, a third thermosensor must exist which detects the temperature of growth/development and mediates thermal adaptation. The rapidity with which the crossover temperature from positive to negative thermotaxis adapts (significant thermal adaptation within 4 hrs at a new temperature) argues against (but does not exclude) the involvement of de novo synthesis of proteins as the mechanism

for adaptation. Alternatively, there are several reports of temperature sensitive enzymes involved in lipid metabolism in microorganisms (see section I).[57,58,69] Such an enzyme could serve as the third thermosensor regulating thermal adaptation. A likely candidate for this role, as discussed in section I, is the Δ-5 desaturase of Dictyostelium. This enzyme introduces the second double bond into both of the endogenous dienoic fatty acids of D. discoideum.[81,99] Growth of amoebae and formation of pseudoplasmodia at 18° versus 23.5°C appears to increase the ratio of dienoic to monoenoic fatty acids in cellular phospholipids. Moreover, the Δ-5 desaturase is particularly active during the last few hours of development, a time which corresponds to the period of greatest sensitivity for thermal adaptation.

Dictyostelium differs from other thermosensory microorganisms which generally move toward and accumulate at some optimum temperature.[132-136] In contrast, pseudoplasmodia of Dictyostelium migrate away from a temperature ≃ 2°C below the temperature of growth and development. The protozoan Paramecium and the bacterium Escherichia coli also differ from Dictyostelium with respect to the behavioral mechanism used for measuring a temperature gradient. Paramecium and E. coli temporally measure their thermal environment. Movement in an "unfavorable" direction results in tumbling (E. coli) or frequent direction changes (Paramecium), whereas movement in a favorable direction continues uninterrupted for relatively long periods of time.[133,134,136] In contrast, Poff and Skokut did not observe frequent direction changes during thermotaxis by pseudoplasmodia of

D. discoideum, and concluded on this basis that thermosensing in Dictyostelium is spatial rather than temporal.[35]

Thermotaxis by the nematode Caenorhabditis elegans resembles thermotaxis by pseudoplasmodia of Dictyostelium in some respects. Although C. elegans exhibits optimum-seeking thermosensory behavior under favorable nutritional conditions, starvation of the organism (similar to Dictyostelium, where starvation precedes development) results in optimum-leaving behavior (migration away from the growth temperature).[135] Caenorhabditis and Dictyostelium also both adapt rapidly to changes in growth/developmental temperature. Adaptation in Caenorhabditis occurs within 2-4 hrs,[135] and in Dictyostelium, as shown in this study, within 2-6 hrs. It is interesting to note that "tracking" of C. elegans at the optimum (growth) temperature appears to involve two thermosensors.[135] One sensor apparently registers temperatures higher than the optimum and directs migration toward cooler temperatures, while a second sensor registers temperatures lower than the optimum and directs migration toward warmer temperatures. Thus, it is the balance of these opposing drives which maintains "tracking" at the growth temperature.[135]

Knowledge of the molecular nature of the thermosensory system in a microorganism is most complete for Caenorhabditis and E. coli. Through the exploitation of mutants of these two organisms, thermosensing has been linked closely with chemosensing. In Caenorhabditis the link between chemosensing and thermosensing is "downstream" from the primary receptor.[135] In contrast, Maeda and Imae have shown that substrate-free, high affinity L-serine receptors

are a necessary prerequisite for thermosensing by E. coli and concluded that this L-serine receptor and the thermosensor are identical.[136]

One final question concerns the selective advantage of positive and negative thermotaxis in D. discoideum. Possibly, thermotaxis is a means of ensuring spore dispersal at the surface of the leaf mulch on the forest floor, the natural habitat of Dictyostelium. The subsurface mulch layer, where D. discoideum amoebae live, would be thermally stable relative to the mulch surface, where the temperature would vary considerably with incident radiation, etc. Given the phenomenon of positive and negative thermotaxis, pseudoplasmodia would move toward the mulch surface whether it were cooler or warmer than the subsurface mulch. Once at the surface of the mulch, the pseudoplasmodia would form sorocarps from which spores would be dispersed by wind, rain and insects. This is similar to the suggestion of Hedgecock and Russell concerning the survival value of thermosensing by Caenorhabditis.[135]

SUMMARY:

I. Homeoviscous Adaptation and Thermotaxis in Dictyostelium discoideum:

Investigation of homeoviscous adaptation in pseudoplasmodial amoebae of D. discoideum, NC-4, is considerably complicated by variables such as bacterial fatty acid composition and the turnover of Dictyostelium fatty acids during development. Furthermore, one would anticipate only small changes in the fatty acid composition of cellular lipids in response to a change in growth/developmental temperature of 5.5°C (23.5° → 18°C). In spite of these complications, phospholipids from pseudoplasmodial amoebae were shown to have a higher level of fatty acid unsaturation at the lower temperature of growth/development. These results are consistent with the "dogma" that microorganisms "adapt" to a lower growth temperature by incorporating more unsaturated fatty acids into membrane lipids.

The results of the GC-MS double label experiment presented here, and the report that lipids of Dictyostelium are catabolized as an energy source during development,[91] tend to rule out the involvement of de novo fatty acid synthesis in the process of homeoviscous adaptation beyond the growth phase of the life cycle. It is more probable that thermal adaptation of lipid composition occurring during development results from the effect of temperature on the metabolic events set into action by the developmental regime. This is an important point, in view of the observation (reported herein) that the last few hours of development are the most critical for thermal adaptation of subsequent thermosensing by pseudoplasmodia.

This time interval corresponds to a period of rapid synthesis of the diunsaturated fatty acids of Dictyostelium as well as the synthesis of sterols and steryl esters.[81,91]

Based on these data, two separate "single enzyme activation" models have been proposed to account for homeoviscous adaptation during development of Dictyostelium. The first model entails the low temperature "activation" of the Δ-5 desaturase of Dictyostelium, which further desaturates 9 and 11 monoenoic fatty acids at the 5 position.[99] The second model involves the low temperature "activation" of a hypothetical fatty acyl transferase, which performs the transesterification of fatty acid from steryl ester to phospholipid. Of these two, the Δ-5 desaturase model is favored since: 1) the enzyme is known to exist; and 2) the greater ratio of dienoic to monoenoic fatty acids in the phospholipid fraction from 18° versus 23.5° pseudoplasmodia could be achieved by a small increase in the activity of this single enzyme. An extensive enzymological study would be required to prove or disprove either of these models. In addition, it should be noted that despite the evidence for homeoviscous adaptation in pseudoplasmodial amoebae of D. discoideum, NC-4, thermal adaptation of thermotaxis is not necessarily a result of the temperature-related changes in fatty acid composition.

II. Membrane Perturbations and Thermotaxis in Dictyostelium discoideum:

The membrane perturbation study sought to alter or eliminate the thermotactic response by pseudoplasmodia of D. discoideum, NC-4, by: 1) blocking new sterol synthesis or precipitating sterols from the cell membrane; or 2) modifying the fatty acid composition of

cellular lipids, and thus membrane lipid fluidity, through fatty acid "feeding". Inhibitors of sterol biosynthesis and function were found to have no effect on thermotaxis. These results may have been due to flaws in the experimental design since: 1) most thermotaxis "assays" were performed on "optimal" thermal gradients, where a small shift of the thermosensory range would go unnoticed; and 2) the concentration of inhibitors of sterol biosynthesis may have dropped to a sub-inhibitory level by the critical time of pseudoplasmodium formation. It is also possible that under the circumstance where normal pseudoplasmodia are formed in the presence of inhibitors, sufficient membrane "repair" has occurred to enable "normal" thermotaxis. Alternatively, a cellular membrane with a low sterol to phospholipid ratio (e.g. the mitochondrial membrane), rather than the plasma membrane, may be involved in thermosensory transduction in Dictyostelium.

The second part of the membrane perturbation study, which entailed the fatty acid "feeding" experiments, was complicated by the requirement for growth of the wild-type strain, NC-4, of D. discoideum in association with bacteria. The most successful experiment, in terms of altered fatty acid composition in pseudoplasmodial amoebae, entailed the prior modification of bacterial lipids through growth at low temperature and fatty acid supplementation. However, the initial goal of the study -- to incorporate more polyunsaturated fatty acids into pseudoplasmodial amoebae of NC-4 -- was not achieved by this technique. K. aerogenes does not synthesize polyunsaturated fatty acids, and incorporated only small amounts of those which were

supplied in the growth medium. Thus, the most extensive modification of fatty acid composition attained for both Klebsiella and Dictyostelium was an exchange of monoenoic for cyclopropane fatty acids. Although such an exchange would be anticipated to have little effect on bulk lipid fluidity,[52] an apparent upward shift of the transition temperature from positive to negative thermotaxis coincided with the increasing ratio of monoenoic to cyclopropane fatty acids in one set of experiments. The shift of the zero transition temperature was slight, and more experiments would be required to ensure statistical significance.

III. Thermal Adaptation of Thermosensing and Negative Thermotaxis in Dictyostelium discoideum:

The study of thermal adaptation of thermotaxis by pseudoplasmodia of D. discoideum, NC-4, was intended to establish whether both growth and development of the organism at a temperature well below 23.5°C (18°C) would result in a complete shift of the range for positive thermotaxis to lower temperatures. The results of the study showed that: 1) pseudoplasmodia exhibit "negative" thermotaxis on temperature gradients with a midpoint several degrees below the temperature of growth and development; 2) the high temperature limit for positive thermotaxis is not shifted down by growth/development of amoebae at a lower temperature; and 3) the temperature of transition from positive to negative thermotaxis is thermally adaptive. These data argue against the single membrane lipid phase transition-as-biothermometer hypothesis for thermotaxis in

Dictyostelium. As an alternative, two two-thermosensor models have been proposed to account for both positive and negative thermotaxis, and the adaptive crossover temperature between these opposite responses.

The first of these models involves separate thermosensors governing the positive and negative responses. The crossover temperature from positive to negative thermotaxis would then result from competition of the opposite responses at that temperature. The alternative model entails one thermosensor which detects a small temperature differential over the entire range of both positive and negative thermotaxis, and a second, adaptive thermosensor (perhaps a membrane lipid phase transition) which abruptly switches the sign of thermotaxis. At present, the data favor the separate positive and negative thermosensor model. The downward shift of the zero-transition temperature from positive to negative thermotaxis on 0.11°C/cm versus 0.22°C/cm gradients (see Figures 3 and 4) may be indicative of different temperature dependencies (sensitivities) of the positive and negative responses. In addition, no evidence for a thermally adaptive lipid phase transition in membranes of Dictyostelium has been found thus far. Further studies of the temperature dependence of positive versus negative thermotaxis, as well as an investigation of the lipid phase transitions of D. discoideum membranes other than the plasma membrane, might establish which of these models (if either) is valid.

In addition to the two thermosensors proposed to explain positive and negative thermotaxis, and the adaptive crossover temperature from one to the other, a third sensor must be included to

account for the phenomenon of thermal adaptation. If thermal adaptation of thermosensing is a consequence of homeoviscous adaptation, the Δ-5 desaturase discussed in section I could serve as the thermosensor for this process. The demonstration of low temperature "activation" of this enzyme would support this proposal.

Two further questions addressed in the study of thermal adaptation of thermosensing in Dictyostelium were: 1) How rapidly can adaptation occur? and 2) What time during the course of development (if any) is most critical for thermal adaptation of subsequent thermosensing by pseudoplasmodia? The three series of temperature-jump experiments intended to answer these questions showed that: 1) substantial but not "complete" adaptation occurs when a temperature-jump is performed at the outset of development (0 hrs starvation); 2) significant adaptation occurs within 4 hrs after shifting to a new temperature, provided that the T-jump takes place at the end of development; 3) the most rapid rate of adaptation occurs during the final 2-6 hrs of development, when pseudoplasmodia are formed from tight aggregates; and 4) a T-jump during the final 8 hrs of development is as effective in producing thermal adaptation of subsequent thermosensing as a T-jump given at the beginning of development (0 hrs starvation). As discussed in section I, the correlation between the period of most rapid adaptation and the period of pseudoplasmodium formation suggests that temperature affects the metabolic events involved in differentiation.

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LITERATURE CITED

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