EFFECT OF TEMPERATURE-INDUCED MEMBRANE LIPID PHASE TRANSITION IN RECIPIENT CELLS ON CONJUGATION IN *ESCHERICHIA COLI* K-12

by

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INTRODUCTION

Conjugation in prokaryotic organisms is a process allowing the transfer of genetic information (deoxyribonucleic acid, DNA) from a cell (donor) in possession of a transferrable genetic element (conjugative plasmid) to a cell (recipient) capable of receiving transferred DNA (46, 156). Although many pre- and post-transfer events of conjugation are now fairly well understood, the actual mechanism by which DNA transverses the cell membrane remains rather nebulous. It would appear that some form of transport system is required, possibly sharing characteristics common with transport systems for cell metabolites. However, the DNA transfer system is obviously confronted by unparalleled constraints. For instance, the conjugative plasmid, F, found in Escherichia coli K-12 is a circular structure of 60 megadaltons (326, 365), 90 to 100 kilobases (kb) in length (3), and is transferred whole in an oriented linear fashion across the envelope barriers of both cells. No other molecule transported by Escherichia coli approaches this dimension. More intriguing still is the nature of adjustment in the DNA transport system which permits transfer to the recipient cell of the whole chromosome, a 1500 megadalton single-stranded length of DNA (47), following integration of the F conjugative plasmid into the host chromosome.

The nature of the cell barrier places further limitations on the design of this conjugation transfer mechanism particularly in the Gram-negative organism such as Escherichia coli K-12 whose cell boundary is composed of two membrane layers on either side of the

rigid peptidoglycan (69); in essence a structure composed of alternate hydrophobic (membranes) and hydrophilic regions (periplasmic space). The cell envelope is generally capable of restricting passage of all hydrophobic molecules (147, 188, 262, 393) and those hydrophilic molecules in excess of approximately 660 daltons (259, 288). Apparently the transfer event requires a cooperative interaction between donor and recipient membranes in order to by-pass the normal restrictions of envelope permeability, possibly through alignment of channels or pores (6) or some site-specific fusion.

Despite the potentially interesting nature of this subject matter, research on the involvement of cell envelope structures in the process of conjugation has been limited. Investigations to date relating cell envelope structure and function to the process of conjugation have involved either (1) the characterization of mutants selected for deficiencies in outer membrane lipopolysaccharide (an outer membrane structural component) and/or outer membrane protein that concomitantly demonstrate decreased conjugation proficiency (6, 93) or (2) the analysis of the mechanism by which isolated lipopolysaccharide and/or outer membrane protein inhibit matings between conjugation proficient strains (6, 93). No reports are known to exist which relate phospholipid composition or membrane bilayer structure to mating proficiency.

In the hope that some insight might be gained into the role of phospholipids and the membrane structure relative to DNA transport during conjugation this investigation was initiated. Specifically,

the role of membrane lipid fluidity on the ability of mating cells to complete transfer of plasmid DNA and establish the plasmid within the recipient is explored. Such an approach is justified by the facts that (1) phospholipid serves as a major component in both the outer membrane and cytoplasmic membrane (283, 351) and most certainly is as likely to be involved in conjugation as are proteins and LPS, (2) certain membrane functions have relatively specific phospholipid requirements (373) and (3) changes in membrane fluidity have been previously demonstrated to effect other transport and membrane assembly processes (75, 77).

The following literature review, in order to serve an adequate base relative to the investigation, is divided into two major sections. The first section consists of a review of (1) the definition and description of conjugation, (2) the nature of the cell barriers and permeability functions of the outer membrane, and (3) the current knowledge pertaining to the role of the cell envelope of donor and recipient in conjugation. The second section consists of a review of those molecular events which define the membrane phase transition phenomenon and its influence on transport and other membrane-associated functions.

LITERATURE REVIEW

I. Conjugation

A. Genetics of Conjugative Plasmids.

Conjugative plasmids of Escherichia coli range in size from 25 to 70 megadaltons (154, 236, 326, 327), the larger plasmids carrying determinants in excess of those required for plasmid transfer and maintenance functions. The smallest plasmids carry sufficient DNA to code for approximately 100 genes (236) but only a quarter of that number have been identified (236). Of these identified genes, nineteen are known to participate directly in the event of conjugation (384) and are collectively referred to as the tra genes . Thirteen are required for the synthesis of the donor cell sex pili (157, 384), and of these, only the traJ (236) and the traA (384, 387) have been implicated as coding for pilus structural subunits. The remainder of the tra genes code for functions that involve DNA metabolism during conjugation; initiating the processes required for either modification of DNA to a form that will permit transfer (mobilization) or for replication of the strand of DNA transferred (conjugative replication) (236, 387).

The remaining genes are involved with vegetative replication and maintenance. Vegetative DNA replication of the plasmid occurs in coordination with the cell division cycle (208, 209) and is distinct from the conjugative DNA replication occurring during transfer. An origin of vegetative replication (62, 242, 304, 385, 386) as well as genes coding for replication functions (384) have been identified.

Genes involved in maintenance of the plasmid within the host cell include those coding for functions which prevent matings between cells with like-plasmids (surface exclusion) (7), and also those with functions preventing two like-plasmids from completing replication within the same cell (incompatibility) (96, 97, 248, 376). Although not properly characterized with any of the above functions, F- conjugative plasmids also possess the capacity to insert (figuratively) the host chromosome into the plasmid through integrative recombination (98, 143). Such is possible through the existence of regions of DNA called insertion sequences (IS) (98, 143). Integration of the plasmid and the host chromosome through any of the numerous plasmid IS sequences permits transfer of the entire host chromosome via conjugative functions of the plasmid. Such donor cells are referred to as Hfr donors; the acronym Hfr referring to high frequency of transfer of chromosomal determinants (91). Reversion of integration may lead to either regeneration of the autonomous and unaltered forms of the plasmid (F) and chromosome or the formation of a plasmid (F') to which some chromosomal genes are linked (F' plasmids carrying those genes linked to the site of F integration).

B. Requirements for Completion of Conjugation.

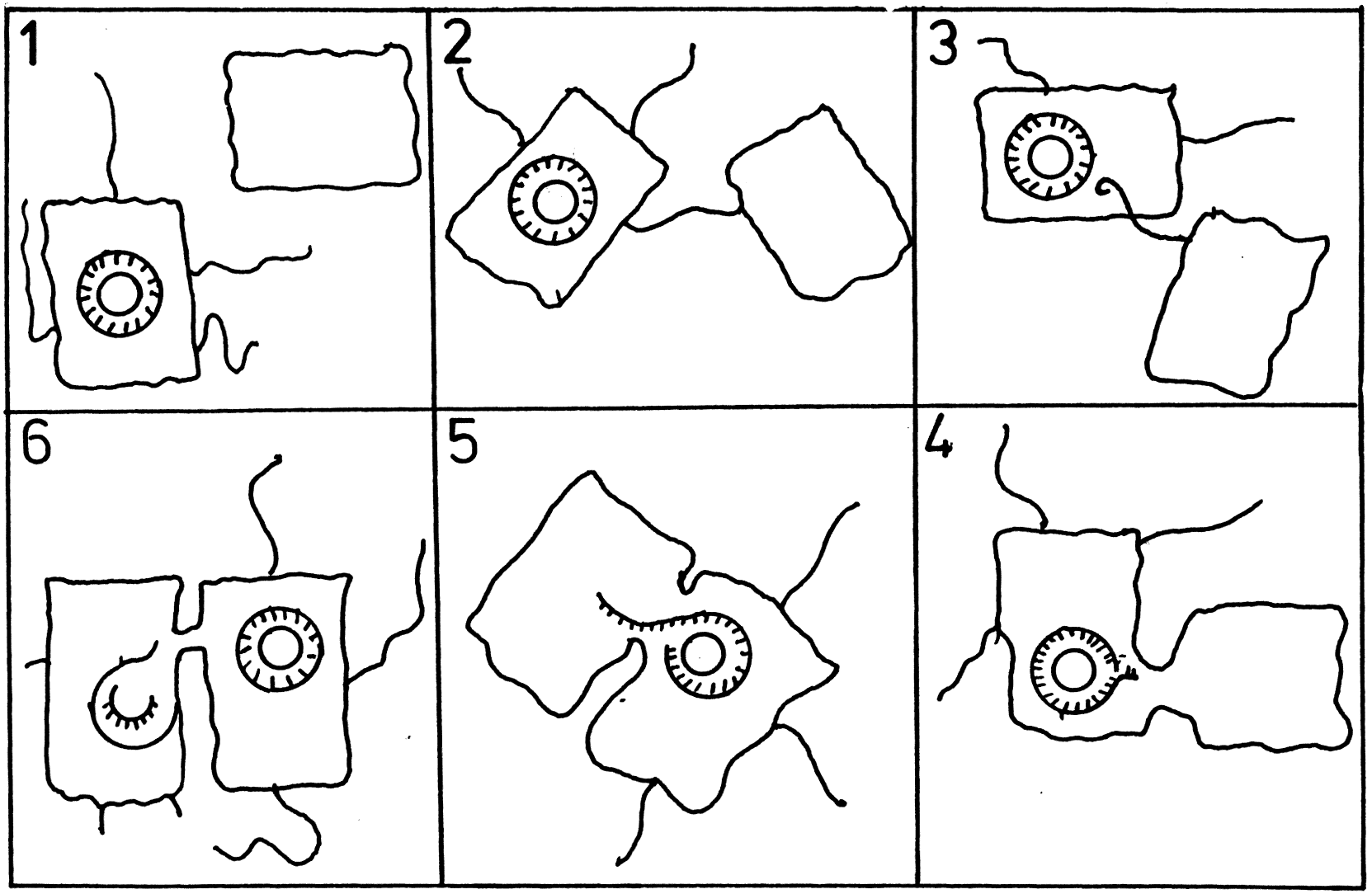
1. Current Terminology. Transfer of a conjugative plasmid requires the completion of a large number of events (many occurring concomitantly) in both cells participating in the mating. Because at least some of the events are discernably sequential and it simplifies

discussion, conjugation is currently defined as the completion of a series of five steps as defined by Achtman and Skurray (16) and Curtiss et al. (93). Combining the different terms currently in use, Curtiss et al. (93) has suggested that the steps in conjugation be referred to as: (1) specific union formation, (2) effective union formation, (3) DNA mobilization, (DNA metabolism required prior to the actual transfer step), (4) transfer of DNA and (5) termination of conjugation. These steps of conjugation are illustrated in Figure 1.

2. Specific Union. A correct orientation between the recipient cell surface receptor and the donor pilus which projects outwards from the donor cell surface is sufficient to initiate specific pair formation. Little or no energy expenditure by either donor or recipient is required (6, 93, 236). The reaction between donor pilus and recipient receptor site apparently requires that the tip of the pilus remain unobstructed, specific union formation being prohibited in the presence of either F-specific bacteriophage, f1 (178, 265, 274), or Zn^{++} (275), both known to adsorb to the pilus tip (274, 362). Additional evidence which suggests that pilus-tip structure is critical to pairing arises from the observation that some donor mutants which possess normal pili as viewed by electronmicroscopy are incapable of entering into pair formation due to mutations apparently affecting the molecular arrangement of the pilus tip (7).

3. Effective Union. This stage is generally considered to be initiated with the formation of wall to wall contact between mating pairs (91, 93). Wall to wall contact is believed to be generated

Figure 1. Schematic Representation of the Steps of Plasmid Transmission by Conjugation in Escherichia coli K-12. (1) Donor cell (containing concentric circles representative of the conjugative plasmid) comes into proximity to the recipient cell. (2) Pilus of donor cell makes contact with receptor site on recipient cell initiating specific union formation. (3) Pilus is withdrawn by donor through some undefined mechanism, pulling the recipient cell surface into contact with the donor cell surface. (4) Initiation of effective union by donor and recipient; membrane fusion likely occurs. Chromosome mobilization is also depicted as occuring in this time frame but it may be actually initiated at an earlier point. (5) Transfer of DNA to recipient cell, normally occurring simultaneously with DNA synthesis in the donor cell as the transferred strand is replicated. (6) Establishment of transferred plasmid DNA in recipient cell requires conversion of the single strand to double stranded DNA, which is then to covalently closed. Information extracted from Curtiss (91).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through the retraction by the donor of the sex-pilus attached to the recipient through the receptor site (93). Failure to retract the sex-pilus, due either to attachment of certain bacteriophage to the sides of the sex-pilus (265, 274) or to certain tra mutations (7), prevents the conversion of specific unions to effective unions as well as the completion of transfer of donor DNA (suggesting that DNA is not transferred through the pilus). Electron micrographs of mating cells suggest that effective union may involve fusion of the cell envelopes (341), a possibility supported by the observation indicating an exchange of envelope proteins between mating cells (93). Expenditure of energy by the donor cell is likely required for the retraction of the pilus, and there is an apparent requirement for the maintenance of membrane potential by the recipient cell as a prerequisite to effective union formation (139) implicating a non-passive role for this cell of the mating pair as well.

The absolute requirement for wall to wall contact between cells participating in conjugation has been questioned by Ou and Anderson based on the observation (276) that the transfer of DNA can occur in the absence of effective union formation. These investigators, using microscopy and micromanipulation, found that paired cells (pili attached to recipient cell receptor site) separated by a distance of up to 3 um were capable of yielding transconjugant clones inheriting either plasmid (F') or chromosomal (Hfr) determinants. The authors, however, recognized that the 6 minutes it took to isolate the first mating pair from a mating mixture was sufficient time to allow for

both mobilization (5 minutes as estimated by Low and Wood (227)) and completion of transfer of approximately 1.2 x 10^8d (60kb) of single stranded DNA; equivalent to either an entire F plasmid or 8 minutes of an Hfr Escherichia coli donor chromosome. Thus, mating cells isolated in what was believed to be the initial stages of specific union may not have been so. In the case of matings with Hfr donor cells, isolated pairs may have included recipient cells which had previously participated in a mating that had prematurely terminated following completion of transfer of those determinants used to determine successful transfer. In matings with F plasmid donors, isolated pairs may have included recipient cells which had already received a plasmid from a previous donor and had yet to express the genes coding for surface exclusion. Finally, with either donor type present, the pairs isolated may have been in the terminal stage of conjugation (see below) and were still physically paired at the time of isolation.

To compensate for such criticisms, Ou and Anderson also studied matings requiring transfer of late chromosomal determinants (8 to 16 minutes distal from origin of transfer) as demonstration of successful transfer. However, these results too may be suspect. Since the assigned transfer time for the markers used are actually statistical averages, it is possible for single cells to transfer at times earlier or later than generally observed. Additionally, the chromosomally integrated F-factor responsible for promoting transfer of chromosomal genes could integrate at a second site allowing earlier transfer of the markers in question. Also, the method used to detect

transconjugants (plating cells on selected media) would allow transfer to occur independent of the observations made by microscopy. Finally it is possible that time of entry (time between initiation of mating and the expression of newly acquired genes by transconjugant) may actually be different for the two procedures. Thus the two isolated cases specifically discussed by Ou and Anderson (276) can hardly be regarded as absolute proof of transfer of DNA in the absence of wall to wall contact. Additionally, the absence of any data suggesting that DNA can pass through the pilus or is in some way associated with the pilus is further reason to doubt that transfer can occur in the absence of wall to wall contact (91).

4. Mobilization of Donor DNA. The step of mobilization of DNA during conjugation has been extensively described in donor cells possessing the conjugative plasmid R64-11 (92, 121, 122, 123) and certain currently described events of mobilization may thus be specific to this plasmid. However, it is very likely that all conjugative plasmids direct a similarly general pattern of functions during initiation of transfer. Initially free in the cytoplasm, the plasmid binds to the donor membrane (92, 122), and an initiator protein, presumed to be the endonuclease encoded by the traI gene on the plasmid (236, 387, 388), then yields a structure permitting the transfer of a unique strand of DNA to the recipient cell (92, 122). Mobilization also requires the presence of a product for which synthesis is rifampin-sensitive, probably an RNA primer necessary for the inititation of conjugational DNA synthesis (92, 122, 243). The

rifampin-sensitive product is not present from 5 minutes to 30 seconds before initiation of mating (92, 122, 243) but has been found to appear within 15 seconds of the mixing of donor with recipient (93, 243). Since very few stable mating aggregates are generated in such a short period and no recipient cell-synthesized soluble factors capable of initiating mobilization in the donor cell have been detected, it would appear that mobilization possibly requires minimal cell to cell interactions (donor pilus: recipient receptor) or undetected soluble factors (93).

5. Transfer of DNA. Conjugational DNA transfer appears to require functions of both donor and recipient cells. Autoradiological experiments (141, 162) suggest that DNA transferred to the recipient cell consists of one strand of DNA synthesized in the donor cell prior to the onset of conjugation regardless of whether the donor is an Hfr type donor (141) or a F^+ donor (162). This observation suggested that new DNA is normally synthesized in the donor to replace that DNA transferred and is also synthesized in the recipient to allow formation of a complement to the newly acquired DNA strand (182). Later investigations of DNA metabolism during conjugation confirmed single-stranded DNA transfer (65, 166) but also demonstrated that transfer of DNA by conjugation is neither dependent on any functions required for vegetative DNA synthesis (33, 121, 238, 364) nor on DNA synthesis (192, 318). In matings with Hfr donors, the initial segment of transferred DNA pairs with its homologue in the recipient chromosome which is required for transfer of the remaining chromosome (273).

The form in which plasmid DNA is transferred to a recipient cell is not as clearly defined. Ohki and Tomizawa (267) reported that the length of DNA transferred by F'gal (λ) donor cells was equivalent to two or three times the length of the plasmid, supporting a rolling circle mechanism of conjugal DNA synthesis (135). The work of Matsubara supports this contention (242). However, reports from the laboratories of Curtiss (92, 121, 122, 123), Vapnek and Rupp (363, 364, 365) and Falkow (120) suggest only single lengths of F factor DNA are transferred in discontinuous rounds separated by 5 to 10 minutes. Regardless of type of donor cell and the form in which DNA is transferred, it is established that the direction of transfer (clockwise or counter clockwise on the chromosome or plasmid) is dependent on the orientation of the origin of transfer within the replicon and the 5'p end always enters the recipient first (267, 315). Thus one donor strain will always transfer the light strand while another donor strain may transfer the heavy strand, but the 5'P end will enter first regardless (267, 315). Asymmetric transfer appears to be the rule, holding true for F plasmid (and Hfr) (363), as well as other incompatibility group plasmid donors (365).

An expenditure of energy by both donor and recipient cells is required for DNA transfer by conjugation. Inhibition of energy metabolism in either donor or recipient results in reduced transconjugant yields (89, 125). Furthermore, as the genetic functions differ between donor and recipient, so the energy functions differ. In matings where an Hfr donor cannot utilize the available energy

source, no alteration in the time of entry of either early or late markers is observed, but the level of transconjugant yield is significantly reduced (89). In matings where the recipient is unable to utilize the available energy source, late marker appearance is delayed and reduced while early marker appearance and transconjugant yield are near normal (89). The data suggested to some investigators (89, 130, 287) that the early stages of transfer were most dependent on energy metabolism within the donor cell, while the latter stages of transfer were most dependent on the recipient cell energy metabolism. Evidence to substantiate this contention exist. In mating experiments employing Escherichia coli minicells [cellular-like forms which lack a chromosome, derived from faulty division of a specific class of mutant (8)] approximately 50 to 80 megadaltons of DNA were transferred to the recipient minicells irrespective of the class of donor strain (Hfr, F', or F) (65, 66). Apparently, even in the absence of active energy metabolism by either donor or recipient, the donor is capable of forcibly transferring to the recipient a segment of DNA equivalent in size to most plasmids, or to the portion of chromosomal DNA near the origin of transfer on either the large plasmids or host chromosome in F' and Hfr donors respectfully (93). The energy source for this event may lie in the potential energy conserved in the structure of DNA itself (384). Transfer of DNA beyond the equivalent of about 3% of the Escherichia coli chromosome requires the expenditure of energy by both cells (93). Evidence also exists suggesting that a membrane potential must be maintained to allow for pair formation and DNA

transfer (139), this contention supported by the observation that non-lethal concentrations of CN^- severely inhibit conjugation (90).

6. Establishment of Transferred DNA. Occurring within the recipient cell during or following transfer, the terminal events of conjugation (establishment) vary depending on the form of the DNA that is being transferred. In matings with Hfr donors in which chromosomal DNA is transferred, successful completion of conjugation depends on the homologous pairing of single-stranded donor DNA with homologous regions within the recipient chromosome (89, 329). Following homologous pairing of the DNA strands, recombination is required to allow integration of the newly transferred DNA strand into the recipient chromosome (61, 63, 273).

In matings where plasmids are transferred, events within the recipient cell have been identified, but not necessarily ordered. These steps are combined under the heading of plasmid establishment as opposed to vegetative plasmid replication, plasmid maintenance. Single-stranded plasmid DNA entering the recipient cell associates with the cell membrane (93, 120, 329). Then, prior to its conversion to a covalently closed circular (CCC) plasmid, the newly transferred DNA is converted to a double-stranded DNA, open circular form (120). Because the steps of plasmid establishment have not been ordered temporally, questions still remain concerning DNA metabolism following transfer and the involvement of other cell structures in the completion of establishment. For instance, it remains uncertain whether circularization of transferred plasmid DNA occurs before or after

conversion to the double-stranded form. Furthermore, it is still not clear whether the single-stranded DNA need retain its membrane association in order to permit synthesis the complementary strand of DNA, as is the case for vegetative replication (105) and with donor plasmid conjugal DNA synthesis (93, 123).

7. Termination of Mating: The final stage of conjugation is disaggregation (4, 6, 236). Based on the difference in time between that required for completion of DNA transfer and that required for cell disaggregation following DNA transfer, it is suggested that the separation of mating cells is an active process which must be initiated upon completion of transfer (2, 6, 236). The disaggregation event is not believed to be due to the expression of newly acquired surface exclusion characteristics in the recipients inheriting plasmids. This is because it is also observed in cells possessing F-factors carrying mutations inactivating surface exclusion (2, 4, 6) and in Hfr x F^- crosses in which the whole F-plasmid is not transferred (2, 4).

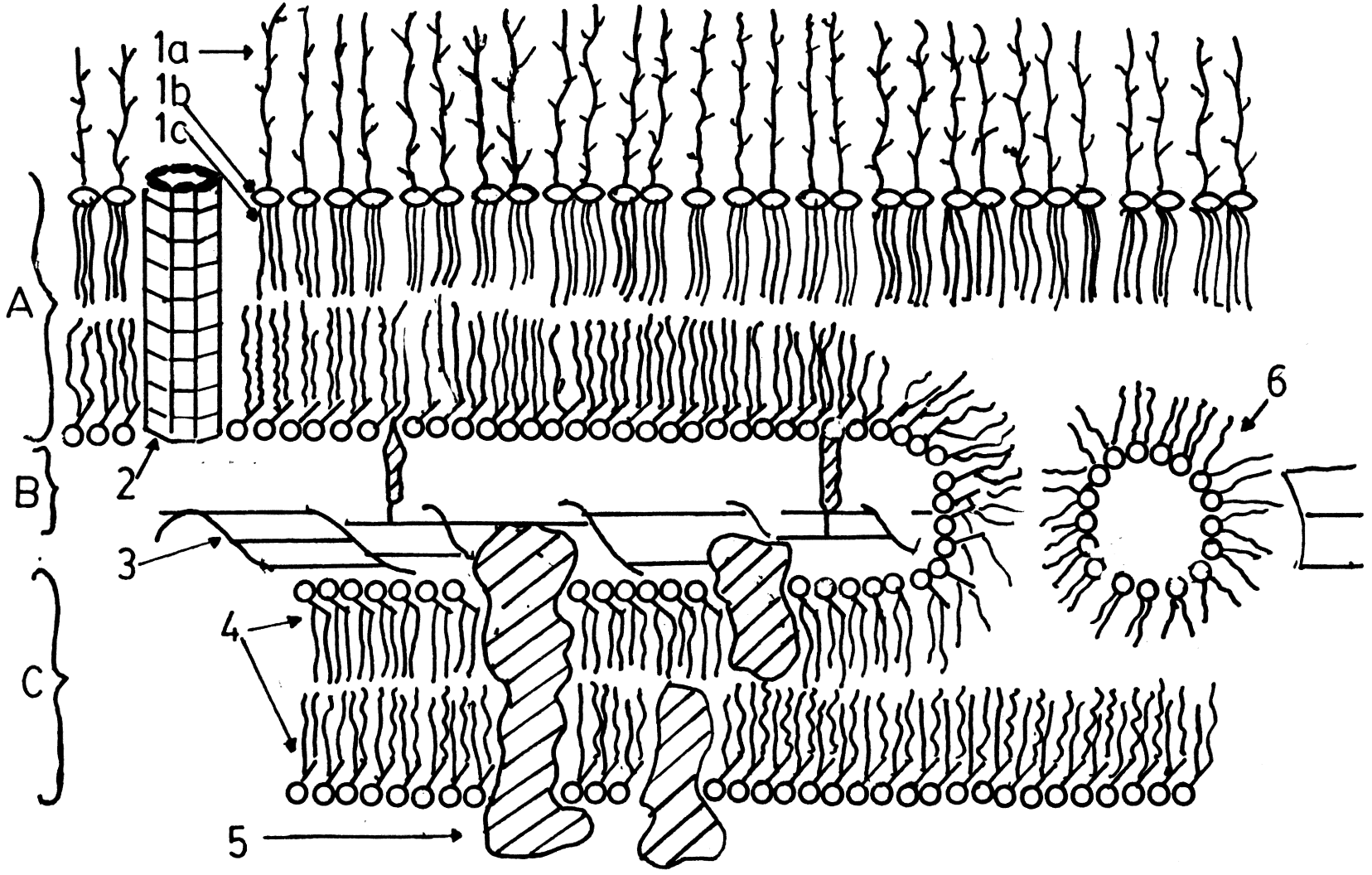
C. Role of Cell Envelope Structural Components in Conjugation.

1. Cell Envelope Structure and Function. In the overall investigation of gene transfer by conjugation in Gram-negative organisms a particularly intriguing area of study is that of the mechanism allowing movement of the macromolecular DNA structure across the complex barrier of the cell envelope. Gram-negative cells possess a cell envelope composed from inside to out of an inner (cytoplasmic) membrane very similar to the cytoplasmic membranes in other organisms (including plant and animal), a rigid wall structure called the

peptidoglycan, and an outer membrane separated from the peptidoglycan by a 22 A wide region (25) called the periplasmic space. The cytoplasmic membrane is likely flush against the peptidoglycan due to tugor pressure, while the outer membrane appears anchored to the peptidoglycan by various proteins bridging the periplasmic space (34). The anchoring proteins are covalently attached to the peptidoglycan and interact with the outer membrane through hydrophobic associations (160, 161) (see schematic diagram of Gram-negative cell envelope, Figure 2).

The two membranes possess similar dimensions and arrangements of subunits (137); however, differences do exist. The cytoplasmic membrane phospholipids contains a higher proportion of unsaturated fatty acids relative to the outer membrane phospholipids (228, 380), and phosphatidylethanolamine comprises a greater fraction of the total phospholipid in the cytoplasmic membrane than it does in the the outer membrane (228). Protein to lipid ratios also vary significantly in the two membranes, being higher in the outer membrane (269, 324). A major component of the outer membrane is lipopolysaccharide (LPS) and clearly distinguishes the outer membrane from the cytoplasmic membrane which has none. LPS is composed of 3 distinguishable subunits, lipid A, core molecule, and O antigen (271) arranged such that the lipid A portion (containing six fatty acid molecules) is embedded in the outer leaf of the outer membrane, anchoring the remainder of the molecule which extends outward into the cell environment (226). The LPS represents 26% of the weight of the

Figure 2. Schematic Representation of Cross Sectional View of Gram-Negative Cell Envelope. The cell envelope is divided into three realms (A) the outer membrane, (B) the periplasmic space, and (C) the cytoplasmic (inner) membrane. In the outer leaflet of the outer membrane lie the LPS molecules composed of (1a) O-specific antigen and core region containing KDO (2-keto-3-deoxymannooctulosonic acid) (1b) the lipid A region, a α-1,6 linked D-glucosamine disaccharide substituted with fatty acids (1c) at hyroxyl and amino groups of the glucosamine. Proteins, such as the porins (2) may span the bilayer while phospholipids are generally restricted to the inner leaflet of the outer membrane. Covering the surface of the cytoplasmic membrane is the sacculus-like peptidoglycan structure (3). The cytoplasmic membrane is composed of phospholipids (4) and numerous proteins (5). A zone of adhesion is represented in the area containing non-bilayer phase lipids (6). Information collected from Costerton et al. (69).


1a
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1c
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4
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6

outer membrane (150) and covers 22% of the total surface area of the outer membrane (254, 339). Because the LPS is localized in the outer leaflet of the outer membrane bilayer (226), 44% of the cell surface in contact with the cell environment is covered by LPS (188) and the rest by proteins with most of the phospholipid restricted to the inner leaflet (78, 339). The major function of the LPS is to generate a permeability barrier to hydrophobic (309, 310, 343) and hydrophilic compounds (258, 259, 343).

Passage of most metabolites across the outer membrane depends extensively on the outer membrane protein composition. Certain proteins (porins) have been identified as the structures responsible for conferring permeability to a wide range of low molecular weight hydrophilic compounds in the absence of specific transport or facilitated diffusion functions (258, 259, 288). In addition to the non-specific mediated passage of small molecules by porins, the outer membrane possesses an additional array of proteins conferring specific transport functions for certain substrates (e.g., maltose, vitamin B12, and iron-chelating siderophores) (187, 188, 197). In addition to transport and permeability functions some of these outer membrane proteins also serve as receptor sites for bacteriophage (187, 197, 305) and colicins (187, 188, 197).

Proteins of the cytoplasmic membrane of Escherichia coli are similar to the proteins of the cytoplasmic membrane of most other

facultative anaerobes in that the functions of most of the proteins appear to be involved in metabolite transport and respiration/energy metabolism.

2. Donor Cell Envelope Function During Conjugation. Recently there has been an increase in interest in the nature of the cellular functions which allow passage of macromolecular DNA through cell envelope barriers during the transfer event. Many of the most current reviews on conjugation have dealt largely on cell surface structures and functions required for successful DNA transfer (6, 93, 236). Currently, the information relative to cell surface functions during conjugation is limited generally to pilus function in the donor and the role of LPS and some outer membrane proteins in the recipient.

In the donor cell, the structure most critical to conjugation is the pilus (38). Alterations in the pilus structure or function generally reduce donorability. No mutants are known which possess altered pili and still function as donor cells (6, 236). Other outer membrane proteins specifically affecting the ability of the donor cell to transfer DNA have not been identified (236). Mutants lacking surface exclusion functions (traS, traT) or the factors responsible for recipient cell death when donor to recipient ratio is high (ilz gene product coding for lethal zygosis) are not altered in their ability to transfer DNA (3, 337); these membrane proteins apparently are not required for the transfer process.

Although the exact nature of the pilus function is still subject to speculation (93), details relative to pilus structure are complete.

Accumulated evidence indicates that the pilus is constructed from the subunit protein component, pilin (12, 37), arranged into two parallel rods generating a structure several microns in length composed of 65-70% α-helix (38, 93) and possessing a central groove of about 20 A diameter (127). Each pilin subunit may be associated with two residues of phosphate (37), one glucose (37) and possibly other carbohydrate components (12).

3. Recipient Cell Envelope Function During Conjugation.

a. Lipopolysaccharide: Many mutations affecting cell surface structures are pleiotropic and, with respect to conjugation, cause the mutant cells to exhibit decreased recipient ability. Among mutations that affect both surface structures and recipient ability are those which alter normal patterns of resistance to certain bacteriophages, antimicrobial agents (colicins) with site of adsorption at the cell surface, and surface disruptive agents such as detergents and dyes (6, 93, 236, 384). Mutants arising from selection for resistance to ampicillin (252), to bacteriophage ϕW (252), simultaneous resistance to ampicillin and bacteriophge ϕW (252), to the single-stranded DNA bacteriophage, ST-1 (306), and simultaneous resistance to bacteriophage T3,T4, and T7 (151, 152) have all been shown to be Con$^-$ and carry alterations in LPS composition when compared to wild type. Many of these Con$^-$ mutants also appear to possess alterations in the outer membrane protein composition (152, 252). Apparently the mutations which affect the LPS composition or structure also result in alterations in the outer membrane protein composition (150, 152, 198,

252, 380). These mutations frequently affect early stages of conjugation; many of these Con$^-$ mutants demonstrating a decreased capacity to form specific and/or effective unions (151, 152).

b. Protein pompA: Another class of Con$^-$ mutants lack the a specific outer membrane protein, pompA (6), coded for by a single structural gene, ompA (159). Mutations at ompA are pleiotropic, generally conferring resistance to bacteriophages K3 (234) and TuII* (159, 161), variable tolerance to group A colicins L and K (99, 100, 101, 147) an inability to form transconjugants with donors carrying F-type plasmids (99, 234, 235), and sensitivity to eosin yellow, ethylenediaminetetraacetic acid (EDTA), and novobiocin (128, 235).

The functional form of pompA is a structure of 28,000 daltons (133) present at about 1 x 10^5 units per cell (133). The protein appears to be linked to the peptidoglycan, and interacts in some manner with the LPS (112, 325). Evidence linking pompA protein to conjugation arises from the finding that mutants possessing altered forms of pompA demonstrate reduced mating proficiency in matings with F-type donors; the degree of reduced recipient ability (50-to 5000-fold) correlating with the degree of disruption in the normal pompA protein structure (151, 234, 235, 237). Finally, the observation that some mutants temperature-sensitive for the production of pompA are also temperature-sensitive for conjugation proficiency further implicate a role for pompA in conjugation (237).

Despite the clear cut involvement of pompA in conjugation with F-type donors (153, 234, 335) the exact nature of the role of pompA

in conjugation remains an enigma. There is rather strong evidence that pompA is not the pilus receptor for although ompA mutants cannot form stable unions in liquid mating mixture as measured by Coulter counter® they can form pairs normally on filters as was determined by electronmicroscopy (5). This suggests a role for pompA in the conversion of specific unions to effective unions (5). If such is the role of pompA, then it must be specific to F-type donors, because ompA variants function as normal recipients in matings with either I-type (R144) (122) or F-like (R100, R136) R-factor plasmid donors (153, 234, 335). Biochemical analysis of pompA also reveals the complex nature of pompA and ompA gene. Purified pompA cannot inactivate phage K3 or inhibit conjugation unless in the presence of LPS (366). Furthermore, eight different mutations in ompA lead to the formation of pompA forms differing in cyanogen bromide fragmentation patterns and degree of membrane incorporation in vivo (159). Through these different forms of pompA it has also been demonstrated that independent modification can occur in three different functions of pompA; colicin receptor function (tolerance), K3 bacteriophage receptor function, and conjugation proficiency (234).

D. A Possible Role for Phospholipids in Conjugation.

Although outer membrane protein pompA and LPS are known to be involved in conjugation, investigations by Achtman et al. (1, 5, 6, 236) and Havekes et al. (151, 152) currently define the role of these components in conjugation to be limited to either pair formation or pair stabilization. If this is the case, then other cell components

such as phospholipids, membrane phospholipid structure, or cytoplasmic membrane protein structures may be responsible for the completion of the functions in conjugation not presently assigned to LPS and outer membrane proteins. In fact some Con$^-$ mutants have been isolated by Falkinham and Curtiss (118, 119) which apparently owe their Con$^-$ phenotype to defects arising in the cytoplasmic membrane (93, 119). The fact that there exists approximately 100 phospholipid species in Escherichia coli certainly enhances the probability of the possible involvement of the cell lipid component in conjugation. Cullis and De Kruijff (81) have suggested that such numbers of phospholipid species within a single organism indicate functions beyond that of the formation of the bilayer structure of the membrane. In fact, a role of phospholipid structure and or metabolism has been recognized in the function of certain membrane-associated proteins (boundary lipids (44, 171, 200, 373)), and transport of substances between inner and outer membranes of Escherichia coli (adhesion zones (25, 146, 271)), as in the passage of DNA during bacteriophage infection of Escherichia coli (25). Relative to a specific role in conjugation for phospholipid metabolism, electronmicrographs of mating cells frequently suggest the occurrence of membrane fusion between mating cells (341). At least one pair of investigators have observed the exchange of membrane proteins (lambda receptor protein) between mating cells (93); such an event likely requiring a cooperative effort by membrane phospholipids.

Studies on the physical chemistry of phospholipids found in the membranes of Escherichia coli indicate that the fatty acid content of

these phospholipids, barring extreme changes in the ionic environment of the cell (81), dictates most phospholipid behavior within the bilayer. Obviously, the investigation of conjugation proficiency in mutants with altered phospholipid fatty acid composition would be advantageous in elucidating any possible effects of altered phospholipid composition and/or structure on conjugation. Mutants of Escherichia coli have been isolated which can neither degrade fatty acids (Fad$^-$) nor synthesize the unsaturated fatty acids (fabA$^-$ or fabB$^-$) required for growth (278, 330, 331, 332). These mutants are capable of incorporating a number of unsaturated fatty acids uncommon to Escherichia coli into the membrane phospholipid when these fatty acids are simply added to the growth medium (330, 331, 332). Some of these unsaturated fatty acids when incorportated into the membranes of these mutants allow the manipulation of membrane lipid physical state while permitting maintenance of the extra-cellular physical parameters (pH, temperature, and pressure) within the range which define normal conditions for other Escherichia coli functions (macromolecular synthesis, transport, and assembly) (108, 145, 277, 283, 317, 347, 351, 391). Using these double mutants (fabA$^-$ or B$^-$, and Fad$^-$), the dependence of motility (225) active transport (277, 279, 391) and membrane-protein insertion (104, 180, 360, 361) on phospholipid unsaturated fatty acid content and the phase state of membrane lipids has been demonstrated.

The remaining section of the literature review serves the following capacity: (1) an introduction to the terms and events that

define membrane phase transition phenomenon in biological systems, (2) a review of the research on phase transition phenomenon in simple phospholipid bilayer systems that have allowed an explanation of the molecular events which occur during phospholipid bilayer and non-bilayer phase transition and, (3) a review of the effects of phase transition phenomenon on physiological processes in cell membranes.

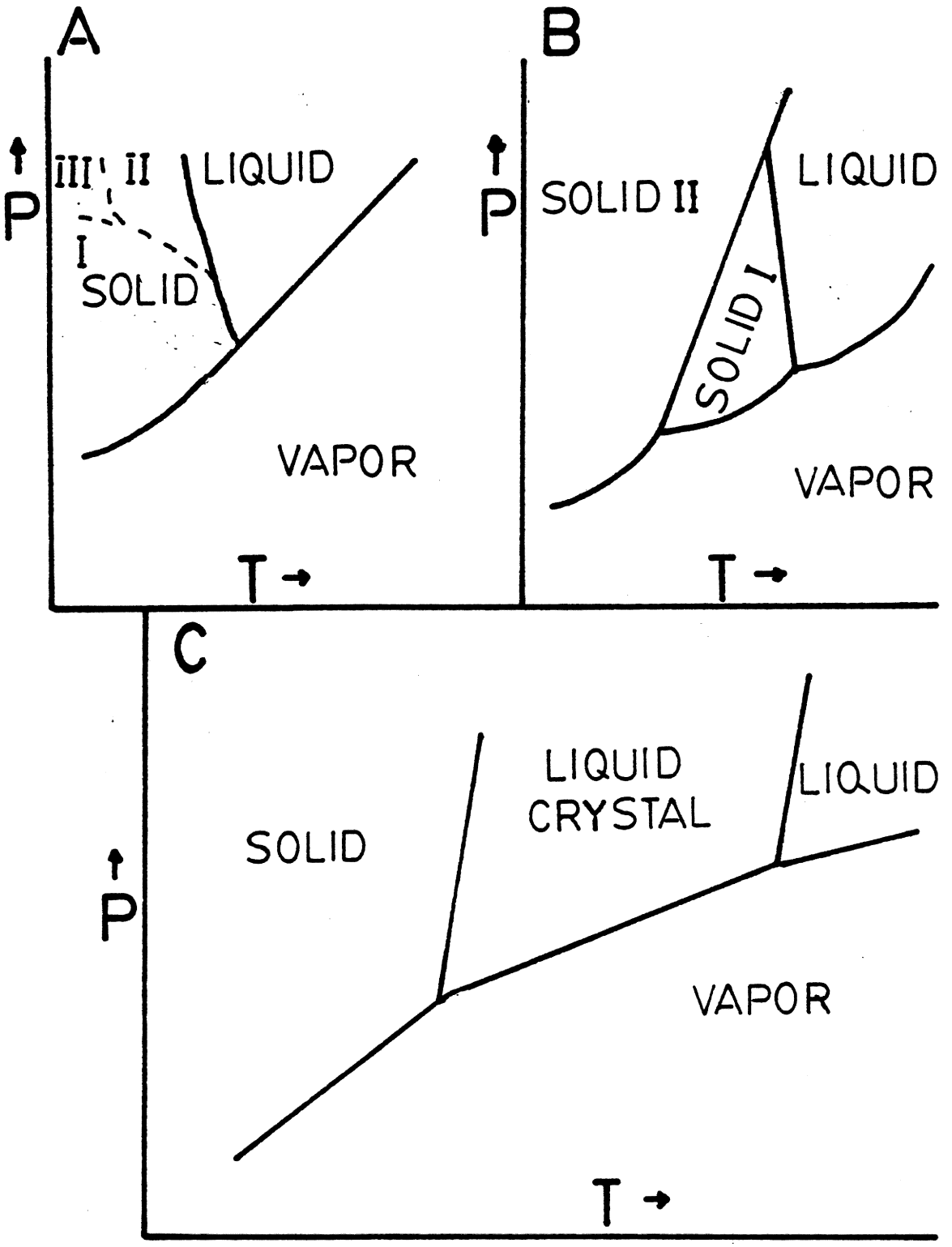
II. Phase Transition Phenomenon

A. The Liquid-Crystalline State of Lipids.

The term liquid-crystalline was first introduced by O. Lehmann in 1904 (215) following F. Reinitzer's discovery of a compound which exhibited the mechanical properties of a liquid and the optical properties of a solid (307). Since that time, a number of organic compounds, including some of biological relevance, have been characterized as exhibiting liquid-crystalline state phase behavior under specific conditions of pressure, temperature, and concentration (53). In all cases, the liquid-crystalline state possesses some optical property of a solid (birefringence, dichromism, or optical activity) in addition to the fluid characteristics of a liquid.

Whatever the substance, the event of a phase transition between the crystalline-liquid (disordered) state and the crystalline-gel (ordered) state is subject to the physical principles which govern all phase behavior (Gibbs Phase Rule). Thus these substances do not differ in this respect from the substances existing in the more common states of matter: solid, liquid, or gas (see Figure 3). Simply stated, the phase transition between disordered and ordered states components,

Figure 3. Phase Transition and Liquid Crystals. Components of a system may exist in a number of different phases, varying with conditions of component number, component concentration, temeperature, and pressure. Critical to the disscussion is the concept that multiple phases for solids and liquids are possible for any single system (gases and vapors may comprise only a single phase). For instance water (diagram A) demonstrates 3 different solid (ice) phases, I,II, and III while Tin (diagram B) can exist in any one of four phases including two different solid phases (solid white and solid grey). In another case (diagram C) it has been observed that some substances can exist in phase(s) which border between solid and liquid, all different liquid crystalline forms. Information collected from Findlay (126).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there are different forms of phase transition (e.g., first-order, second-order and 3/2 types). In some systems where the components are long-chain molecules (alkanes, hydrocarbons, phospholipids) the transition between ordered and disordered states is marked by a complex transition which only approaches the first-order transition observed in simply structured substances (389). This form of phase transition can best be defined statistically (257).

B. Pure Single Species Phospholipids in Non-Aqueous Systems.

Pure, single species phospholipids generally demonstrate (when in the absence of water) transition to isotropic liquids at relatively high temperatures, for example phosphatidylethanolamines and phosphatidylcholines having melting points near 200 C (51). These values are relatively independent of fatty acid chain length or degree of unsaturation and would appear to be due to ionic associations between phospholipid head groups (51). That ionic head group interactions could so strongly affect the melting temperature is indicated by the fact that a large differential occurs between the melting temperatures of free fatty acids and their salts; the melting temperature of stearic acid is 69.7 C while the sodium salt of stearate has a melting point of 300 C (51).

Between solid and isotropic liquid states, pure phospholipids pass through a number of intermediate disordered (liquid-crystalline) states in which the bilayer configuration is maintained while the fatty acid chains become increasingly disordered, no longer perpendicular to the the plane of the bilayer (43, 54, 55, 57).

Though phase transitions between these states (ordered to disordered, disordered to disordered) are not first order transitions as observed with substances such as water (in liquid to ice or liquid to gas transition) or inorganic crystals (257), pure one component phospholipid systems do demonstrate single narrow temperature range phase transition (167, 292).

C. Pure Single Species Phospholipids in Aqueous Systems.

1. Bilayer Structures. With the addition of water to a system previously containing only phospholipid, the bilayer structure is generally maintained and the temperature of endothermic phase transition (ordered to disordered) is decreased (51, 56). Eventually the phase transition temperature, T_t, reaches a limiting value when the system is composed of between 0 and 20%. At this point, the lipids are in the form of liposomes (39) the preferred configuration for many phospholipids in the presence of water (52, 230), and the transition temperature becomes independent of the further addition of water (51, 56).

Formation of liposomes through the addition of water to pure phospholipids is characterized by penetration of water into the polar regions only (56), and is accompanied by a decrease in the thickness of the lipid bilayer resulting from an increased tilt of hydrocarbon chains with respect to the lamellar plane (345). The presence of water apparently results in the prevention of bilayer interaction (117, 290). More importantly, water may help maintain the bilayer structure when hydrocarbons are in the fluid state, preventing

conversion of phospholipids to isotropic liquids. It is found that lipid bilayers in the presence of excess water undergo smaller changes in entropy (107, 290) and correspondingly smaller changes in the volume per CH_2 group (257) relative to pure alkanes upon the initiation of a phase transition. Apparently, the ordered to disordered state transition of alkanes is completely isotropic, while the phase transition of phospholipid bilayers in the presence of water are anisotropic. The anisotropy of the transition results from the fact that the phospholipid head groups are anchored at the aqueous interface (257) thus limiting the increase in molecular volume of phospholipids from 48 A^2 to 70 A^2 upon phase transition (56, 291).

Furthermore the CH_2 groups proximal to the phospholipid head group are restricted in their motion, generally remaining oriented perpendicular to the plane of the bilayer, while only the distal CH_2 groups are allowed to exhibit greater motion (172). Hydrocarbons, thus, have a lower degree of mobility than do n-alkanes above T_t (172). What little change there is in phospholipid volume and motion is absorbed by the bilayer through lateral expansion and transverse thinning of the bilayer (56, 219). No disruption of the bilayer structure occurs.

In the presence of water, the interaction between phospholipid headgroups is minimized and thus the effect of this type of interaction on phase transition temperature is greatly neutralized. Remaining phospholipid headgroup interactions are very weak, limited to electrostatic (dipolar and monopolar), excluded volume, weak hydrogen

bonding between carbonyl groups of the fatty acids at the attachment site to the glycerol backbone of the phospholipid, and non-specific van der Waals interactions (257). Combined or separately, these factors alter the phase transition temperature within biologically significant range (245, 257), but the overall change is generally less than observed from the introduction of one carbon-carbon double bond or the addition of a methylene group to the hydrocarbon chain (257).

Once water has penetrated into the polar regions of the bilayer its influence on additional membrane phase transition phenomenon is limited (257) possibly conferring only a change in surface energy with the increased exposure of the hydrocarbon chain upon transition from ordered to disordered state (257, 344). This conclusion is based on the observation that changes in temperature over the range required to induce lipid bilayer phase transitions do not alter the bilayer structure, thus indicating that hydrophobic interactions are unperturbed by the generation of a phase transition in the region of the hydrocarbon chains (257). Because the transition event is restricted to the hydrocarbon-hydrocarbon interactions (thus excluding hydrocarbon-water interaction) the transition is referred to as a thermotropic transition, the term indicating that the transition involves only the single component, hydrocarbon. Lyotropic phase transitions involve multiple components system, such as would be the case if water did interact with the hydrophobic regions of the lipids during a transition event.

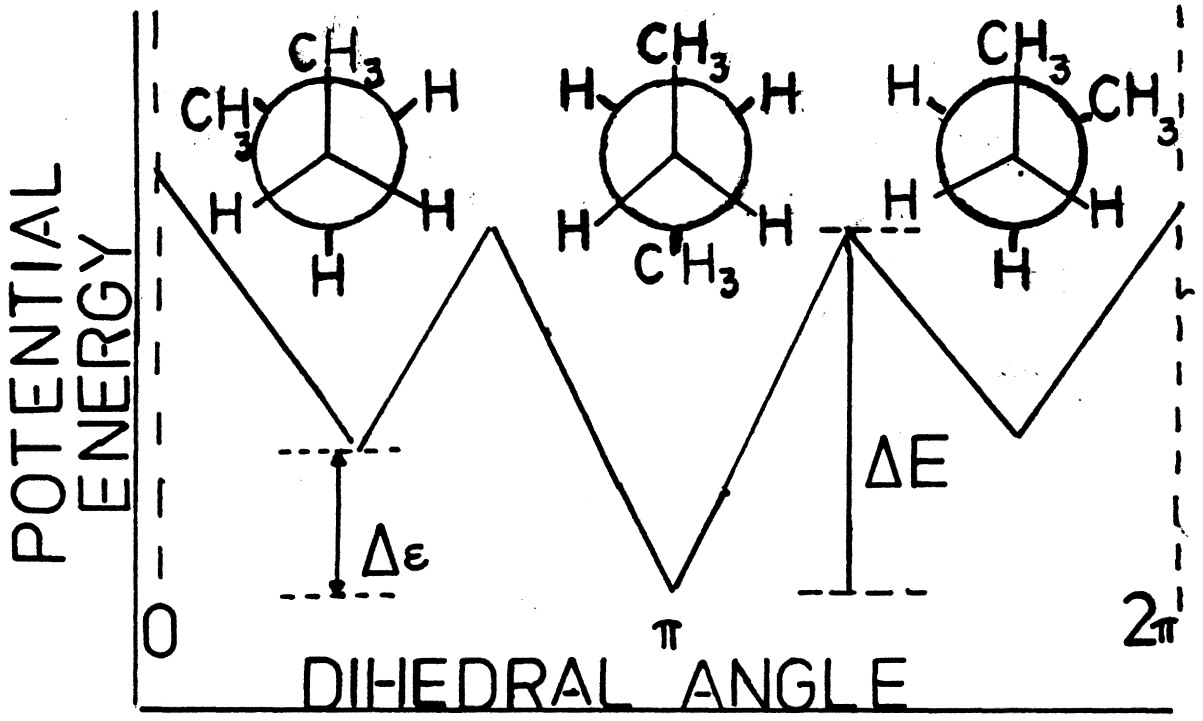
With the reduction in the importance of the phospholipid head group and water on phase transition temperature, the hydrocarbon chains (52, 56, 356) and their position on the glycerol backbone (102) become the significant factor in determining the phase transition temperature in bilayer phospholipids within an aqueous system. Calculations indicate that the heat of transition of the hydrocarbon chains represents 95% of the total heat of fusion for the phospholipids in an aqueous environment (55, 290).

Investigations by Tardieu et al. (345) have yielded information which allows description of the phase transition event in phospholipid hydrocarbon chains on the molecular level. Below the phase transition temperature, the hydrocarbon chain bonds possess minimal free energy and exist only in the trans configuration. With all chains in the trans configuration (Figure 4), neighboring chains have segments in parallel allowing phospholipids to pack into an average area per molecule of 48 A^2 (53, 291) versus the 70 A^2 found for phospholipids at temperatures above T_t. Simultaneous with the packing event, van der Waals interactions between hydrocarbon chains are initiated (53, 291).

Conversion to disordered state from the ordered state requires a cooperative event between hydrocarbon chains, eventually overcoming the attractive interactions arising from their ordered arrangement (257, 312). Increases in temperature allow individual bonds to acquire energy sufficient to allow rotation of the bonds into the higher energy gauche conformation (see Figure 5). This rotation into the gauche state is, however, resisted by chain interactions. Obviously, the

Figure 4. Stable Conformations of a Hydrocarbon Chain: Transition Between Potential Energy States as a Function of Dihedral Angle. In the upper diagram of Figure 4 the center structure depicts the low energy state trans conformation of a simple 4 carbon chain butane molecule. To the left and right of the center structure are depicted gauche conformations which also possess low potential energy levels but still exceed the potential energy level of the trans conformation by a value equivalent to $\Delta\varepsilon$. Rotation from one low energy state to another requires formation of eclipsed conformations which are very unstable having potential energy levels exceeding that of the trans rotational state by a value of ΔE. Thus trans and gauche states predominate in simple carbon chain molecules.

The lower diagram in Figure 4 demonstrates the variety of cones of revolution available to the third and fourth bonds of a simple 4 carbon chain molecule as well as the the stable conformations (1 trans and 2 gauche) of the third carbon bond. In an 18 carbon chain saturated fatty acid there are 380,000,000 (3^{18}) stable conformations, only one of which is all trans. Information extracted from Cowie (70).


POTENTIAL ENERGY
CH3
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CH3
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CH3
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CH3
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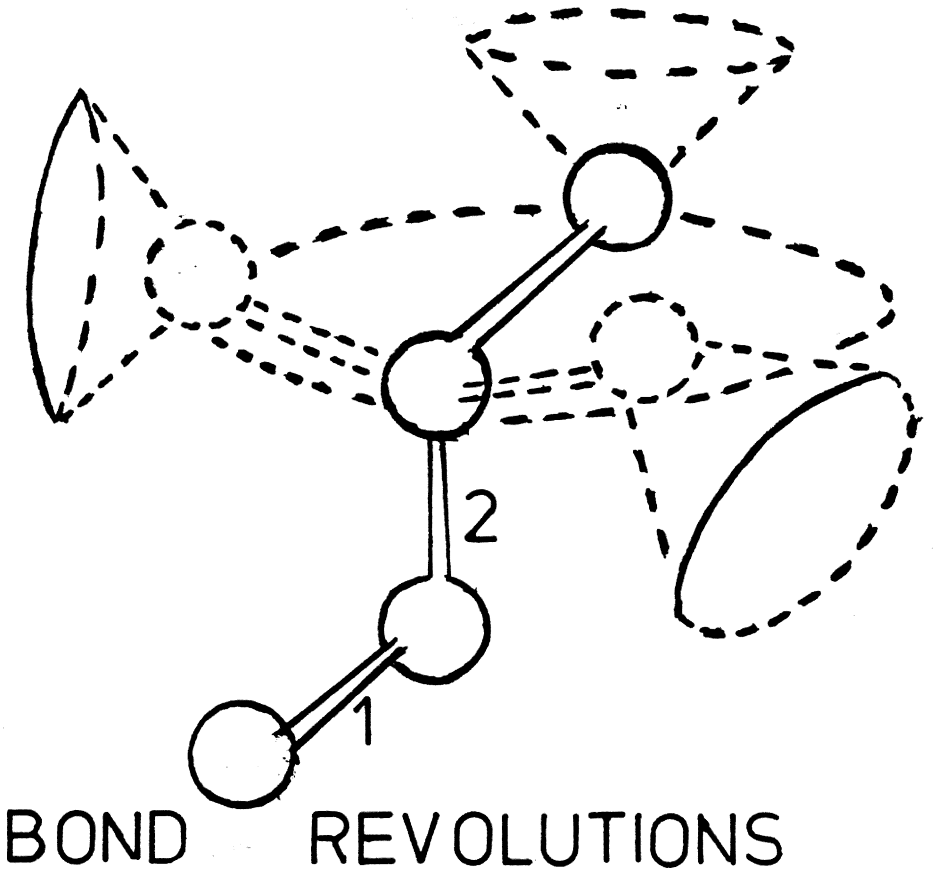
DIHEDRAL ANGLE


2
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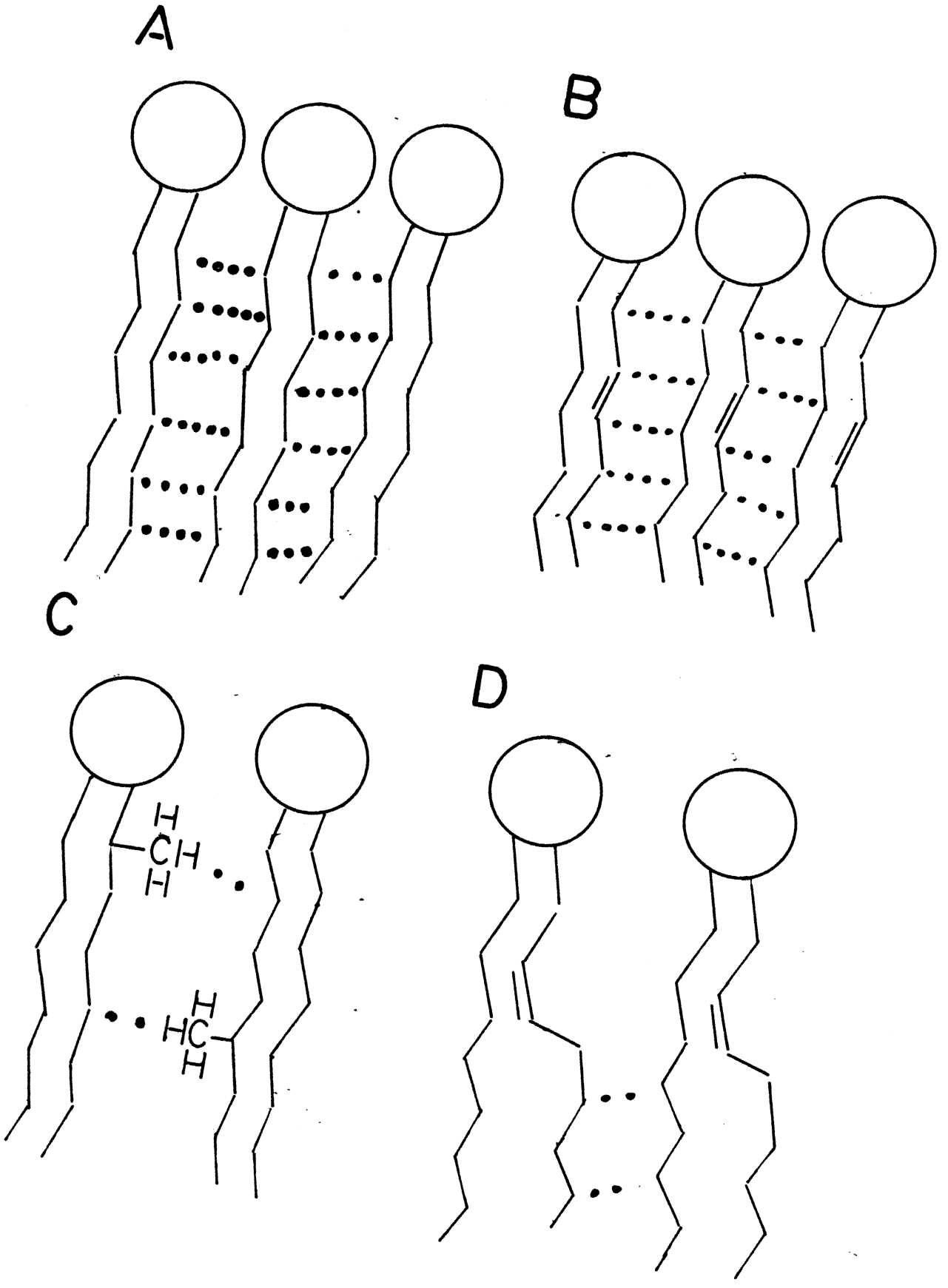
BOND REVOLUTIONS

Figure 5. Molecular Configurations of Low Energy Phospholipids. When low energy state predominates, rotation between various potential energy states is restricted and eventually the majority of methylene groups are in trans conformation. The symmetry of an all trans (A) conformation allows the phospholipid molecules to pack in closely and initiate the transition from the disordered (liquid-crystalline) to the ordered (crystalline) phase state. Unsaturated fatty acids possessing trans conformation double bonds (B) do not disrupt the ability of low energy state fatty acids from assuming the associations between molecules that result in the formation of the ordered phase state. The presence of either side-chain methyl groups (C) or cis double bonds (D) reduce the ability of low energy state fatty acids to associate in a manner conducive to the formation of the ordered phase state. Information extracted from Cronan and Gelmann (75).


A
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closer the packaging of phospholipid groups, the more numerous the inter-molecular interactions and, thus, the more resistance there is to conversion from ordered to disordered state. The greater the resistance in the conversion from ordered state to disordered state the higher the temperature of transition.

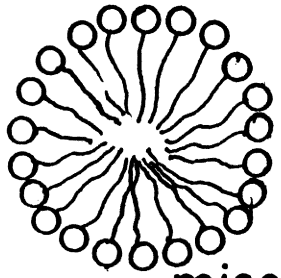
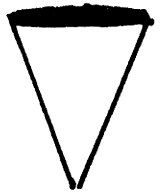
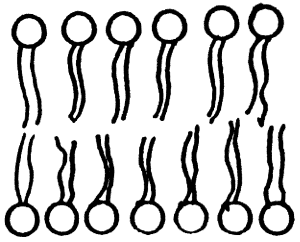
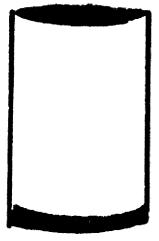
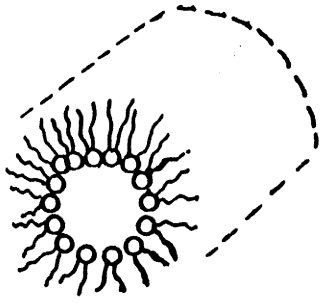
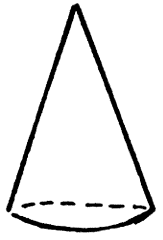
Lengthening the hydrocarbon chain or the presence of a trans double bond configuration results in an increase in the temperature of transition (51, 75). The presence of cis double bonds, shortening of saturated fatty acid chain length, the presence of side chain methyl groups (75) or a shortened fatty acid at position 2 on the glycerol backbone (191) lower the phase transition temperature (see Figure 5).

The differences between aqueous and non-aqueous systems of phospholipids is thus apparent. Pure phospholipids may exist in a number of fluid states including and between solid and isotropic liquids states. Addition of water reduces the number of observable physical states eliminating the occurrence of isotropic liquid state and pure solid along with some of the disordered states (except possibly under extreme conditions). The observed phases in aqueous systems are generally restricted to those phases dictated by the energy states of the hydrocarbon chain and the small interaction between head groups. Overall, simple aqueous phospholipid systems serve as appropriate models in defining the phase transition events in biological membranes and in setting appropriate parameters for investigating phase transition events in biological systems.

2. Non-Bilayer Phase of Hydrated Phospholipids. The bilayer arrangement is but one of the many phases available to phospholipids in aqueous systems. Among non-bilayer phases are included the hexagonal H_I and H_{II} phases, the micellar phase, and the rhombic and cubic phases. Many phospholipids actually prefer the hexagonal H_{II} phase over the bilayer phase including unsaturated cardiolipins (when in the presence of Ca^{++} ions) and phosphatidylethanolamines (81). Transition between bilayer phase and non-bilayer phase (bilayer -hexagonal transition) generally occur within 10 C of the high temperature end of the hydrocarbon order to disorder transition (81).

Because phosphatidylethanolamine and cardiolipin are major species of phospholipids in most biological membranes and, of particular interest to this study, comprise upwards of 90% of the total phospholipid composition in Escherichia coli (71, 299), such non-bilayer phase structures may be biologically significant. Formation of non-bilayer phases in biological membranes might require the availability of localized concentrations of either phosphatidylethanolamine or cardolipin possessing unsaturated fatty acids in both position one and two on the phospholipid glyercol backbone. Although the cellular mechanism by which such a condition might be met is unknown, recent evidence indicates that such non-bilayer phase lipids do exist in membranes of endoplasmic reticulum (204, 342), red blood cells following treatment with fusogens (84), and the cytoplasmic membrane of Escherichia coli (40). See Figure 6 for more detail on the nature of non-bilayer phase lipids.

Figure 6. Non-Bilayer Phases of Phospholipids. Some lipids have a tendency to exist in phases other than the bilayer phase. Overall molecular structure of the component phospholipids dictates this preferred phase. Lipids and detergents which possess head groups which occupy a large area relative to the area swept out by the associated fatty acids generally prefer a micelle conformation. Phospholipids which contain head group structures that fill an area equivalent to that of the associated fatty acids generally prefer the bilayer conformation. Phospholipids with relatively small head groups compared to the area occupied by associated fatty acids prefer the H_{II} phase tubular structures in hexagonal array. Information extracted from Cullis and De Kruijff (81).

LIPID	PHASE	COMPONENT FORM
lysophospholipids detergents	 micelle	
phosphatidylcholine sphingomyelin phosphotidylserine phosphatidylglycerol	 bilayer	
phosphatidylethanol- amine (unsaturated) cardiolipin - Ca^{++} phosphatidic acid - Ca^{++}	 H_{II}	

D. Binary Phase Lipid Systems, Ideal Mixtures.

Unlike the sharp single transitions observed in systems containing a single phospholipid species (56, 160, 174, 293), systems composed of a mixture of two phospholipids generally exhibit a broad phase transition over a temperature range, ΔT (Figure 7). The broad temperature range transition is characterized by a high temperature characteristic, T_h, and a low temperature characteristic, T_l, which (respectfully) define the onset and completion of the phase transition as the system passes from disordered to ordered states (59, 129, 277). The mid-point of the curve is defined as the temperature of transition, T_t (59, 129) see Figure 7).

For a binary phospholipid system to exhibit ideal phase behavior as defined in physical chemistry text (126) the system need be composed of two pure phospholipids of the same species possessing very similar hydrocarbon composition (56, 245). Plotting temperature of T_h and T_l versus the concentration of one of the two components (pressure constant), of such a system results in the construction of a phase diagram similar to that schematically represented in Figure 8. The upper curve is referred to as the liquidus curve and the lower curve is referred to as the solidus curve, each point on these curves representing, respectfully, T_l and T_h for a particular mixture of phospholipids as defined by the abscissa. For instance, in Figure 8 the points a and c on line xx' respectfully represent T_l and T_h for

Figure 7. Schematic Representation of Phase Transition in Membrane Lipids. Membranes with disordered phase lipids begin the transition to ordered phase as temperatures pass through T_h. Transition is complete when the temperature reaches T_l. The temperature which results in 50% conversion of the lipids from one phase to another is referred to as the temperature of transition, T_t. Temperatures between and including the temperatures for the onset and the completion of transition cover the temperature range of transition, ΔT. Note that lipid transition is not first-order as is the case for more common substances, hence the broad temperature range of transition. Information extracted from Overath and Thilo (277).

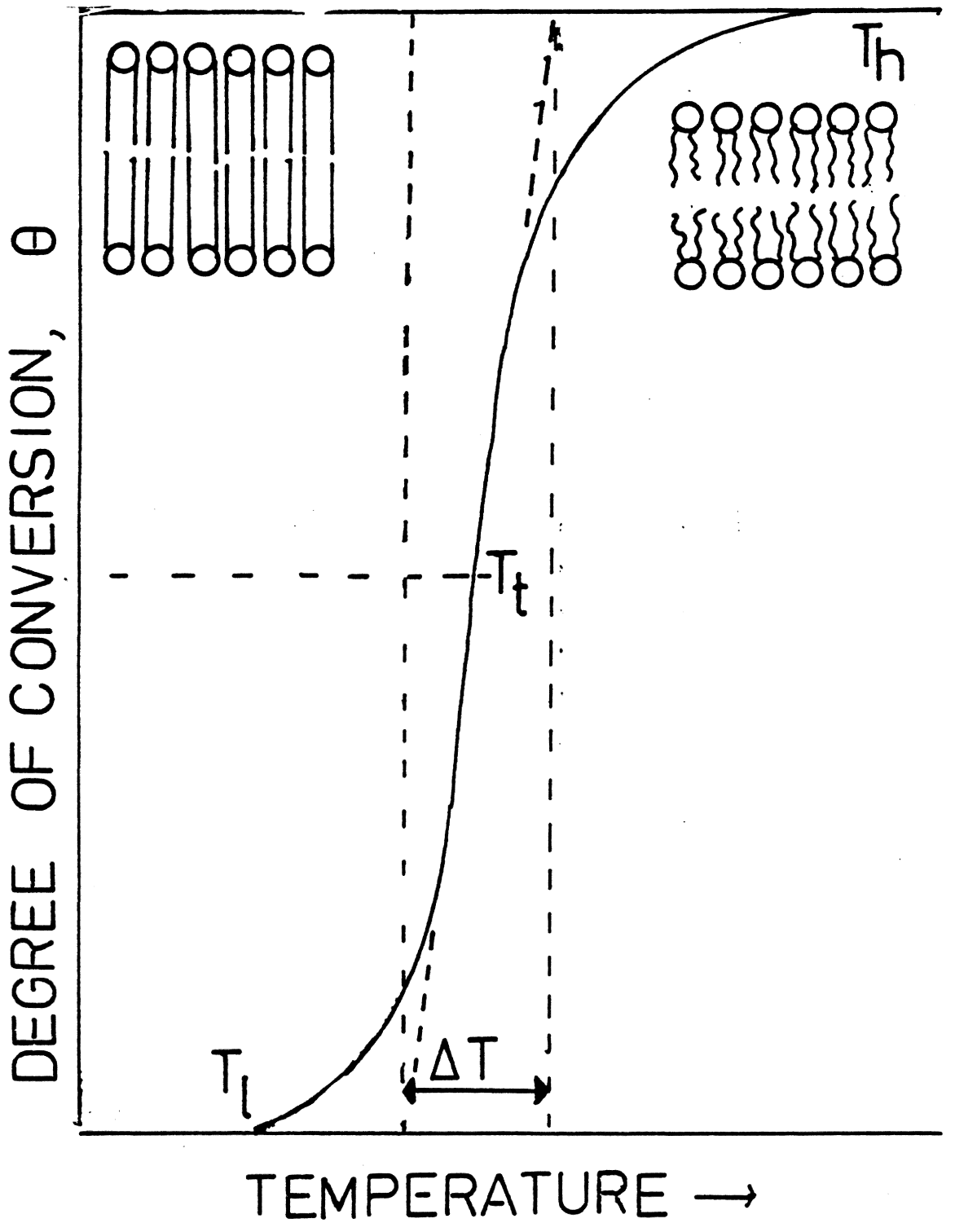
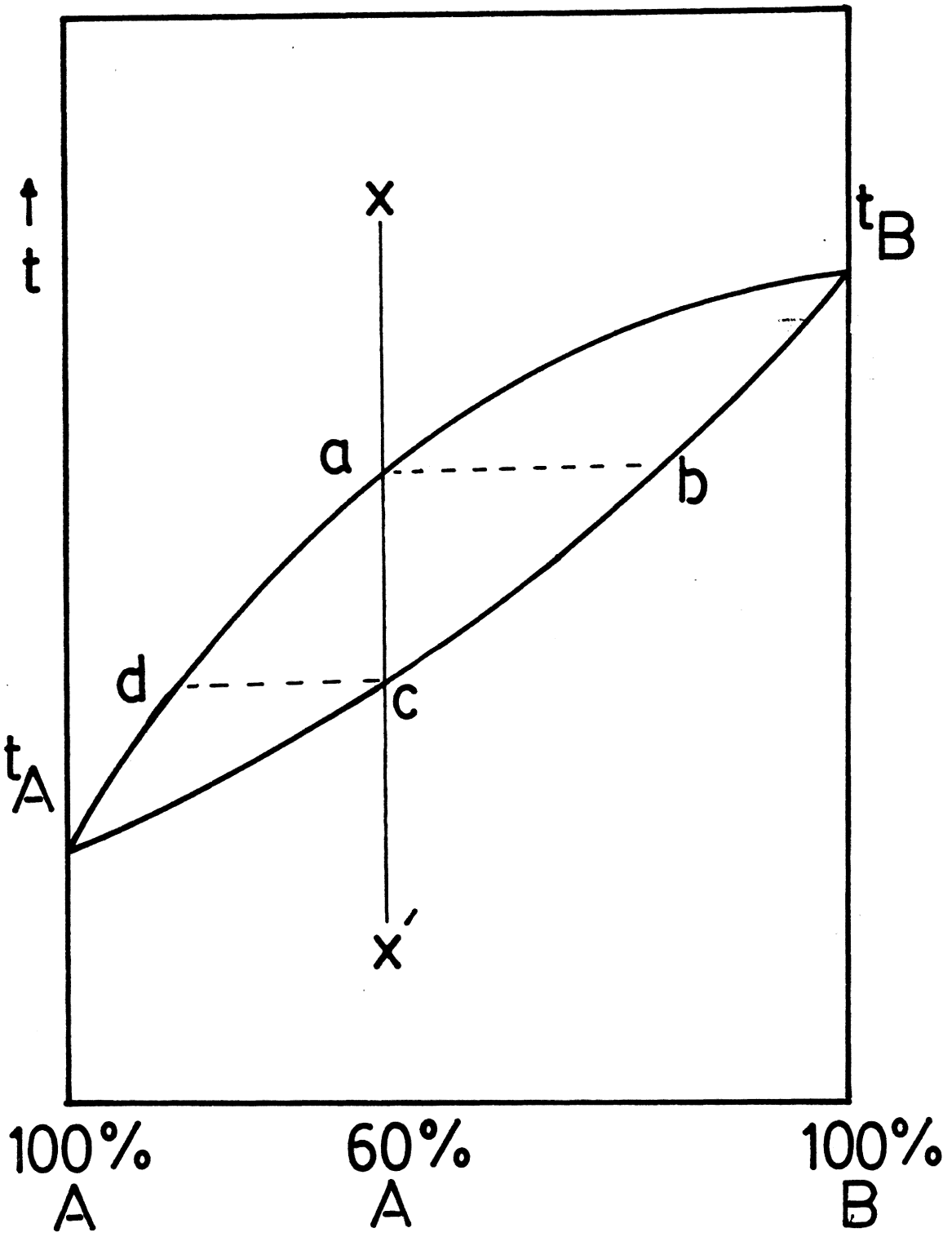

DEGREE OF CONVERSION, θ
TEMPERATURE →
T_h
T_t
T_l
ΔT

Figure 8. Phase Diagram for a Two Lipid Component System. See text for explanation. The symbol t_A represents the temperature of melt for pure component A and the the symbol t_B represents the temperature of melt for pure component B. Information extracted from Findlay (126).


t
t
x
t_B
a
b
d
c
t_A
x'
100%
A
60%
A
100%
B

a phospholipid mixture composed of 60% component A and 40% component B. The distance between a and c representing the range of the transition, ΔT.

Between the range of temperatures T_h and T_l which define the phase transition of a system of mixed phospholipids, it is observed that the relative concentrations of the two phospholipid components differs between the two phases though the two phases are in equilibrium. At any given temperature within the range of transition, the lower temperature of melting phospholipid will demonstrate a greater concentration within the disordered phase while the higher temperature of melting phospholipid will demonstrate a greater concentration in the ordered phase.

Using a phase diagram the composition of both phases at any point in the transition can be determined. For instance, in Figure 8, determination of the composition of the disordered phase during transition is initiated by first drawing a line perpendicular to xx' at the level of the ordinate which corresponds to the temperature, and second by determining the point of intersection of this same line with the liquidus curve. Finally, a vertical line through this point, perpendicular to the abscissa, defines the amount of component A in the disordered phase as that quantity of A defined by the absiccisa at the intersect of this line and the abscissa. Similarly, the composition of the ordered phase may also be determined. A line perpendicular to the ordinate corresponding to the temperature will

intersect the solidus curve. A line through this point on the solidus curve that intersects the abscissa perpendicularly defines a point on the abscissa which represents the quantity of component A in the ordered phase.

Apparently then, as an ideal system of two phospholipids is passed through a phase transition the composition of the two phases will not be constant. At the temperature of point a in Figure 8, the ordered phase will be composed of phospholipids in the proportions of A and B indicated by point b. As the temperature is lowered further there will be a change in the composition of the ordered phase which is represented by the curve bc. Similarly, the changing proportions of A and B in the disordered phase with decreasing temperature is represented by the curve ad. Conversely, heating an ideal system in the all ordered phase through a transition to an all disordered phase should result in a reversal of the above events. At the temperature of c, two phases appear, the liquid phase of composition d. Upon further heating, the change in the composition of the liquid phase is represented by the curve da and the change in the composition of the ordered phase is represented by the curve cb.

It is apparent that the broad temperature range of transition, ΔT, observed in mixed lipid systems results from the fact that the temperature of solidification or liquification upon cooling or heating, respectfully, will not remain constant through the completion of transition but will change relative to the changes in composition of the phases present in system.

E. Binary Phase Lipid Systems, Non-Ideal Systems and Phase Separation.

Calometric and electron spin resonance studies of phase transition in binary phospholipid systems indicate that ideal phase transition behavior arises only under very strict conditions of phospholipid composition (56, 245). Phospholipids must be of the same species (same head group structure) and neither lipid can possess hydrocarbons differing in length from that of the other phospholipids by more than two CH_2 groups. These restrictions define phospholipids which individually do not differ in melting temperatures by more than a few degrees centigrade (56, 245).

Binary phospholipid mixtures which fail to meet the structural restrictions set for ideal systems, are characterized by two separately identifiable temperatures of transition, each indicative of the transition of one of the two component phospholipids (58, 245, 292, 293, 328). Such phase transitions result in the segregation of the lipid components and the system is devoid of regions of mixed ordered and disordered structure. Such segregation of components is referred to as lateral phase separation (195, 328).

The lateral phase separation is accompanied by large phospholipid density fluctuations (175, 286) resulting in mismatched packaging of phospholipids along the interfacial regions between the two phases (240). The formation of short lived pores in these interfacial regions occurs despite the rapid movement of individual lipid molecules (approximately 1×10^8 cm^2/sec) (103, 199, 316). These gaps between

phospholipid phase regions appear to have some physiological significance, resulting in vesicle component phospholipid susceptibility to phospholipase A2 at T_t (272) and increased vesicle permeability to Na^+ (286), K^+ (144, 181), Cl^- (260), Rb^+ (144), glucose (179), β-galactosides and β-glucosides (223), erythritol (144) and 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO)-choline (240). Furthermore transport of K^+ by the ionophore valinomycin is increased under conditions where phase separation is induced in membrane vesicles indicating accelerated trans-membrane motion for this molecule (32, 201, 392). Possibly relevant to membrane protein assembly are the observations which indicate that lateral phase separation in membrane vesicles allows an increase in insertion of membrane proteins acetyl β-casein A (289), bacteriophage M13 coat protein (381), tubulin (194), and serum apolipoprotein A-1 (295)

F. Non-Bilayer Phases in Binary Lipid Systems.

As previously indicated, many of the phospholipids found as predominant species within the majority of biological membranes have a predilection for non-bilayer phase configurations [Figure 6,(81)]. Obviously, since the bilayer is the primary array displayed, there must be some factors responsible for stabilization of the bilayer phase suppressing the tendency of these phospholipids to enter the non-binary phase states. Phosphatidylcholine, phosphatidylserine, phosphatidylglycerol, or cardiolipin in the absence of Ca^{++} all are individually capable of stabilizing bilayer configuration when present in concentrations near 20 to 30% of the total lipid, in a system

otherwise composed of phosphatidylethanolamine (81). Cholesterol may also act to stabilize bilayer phase under limiting conditions (82).

G. Interaction of Non-Lipid Components with Bilayer Lipids and Their Effect on Membrane Lipid Phase Transition.

The association of protein with membrane phospholipid may assume more than one form. Proteins may bind to the surface, bind with partial insertion, or become embedded in the membrane (intrinsic protein) with surface exposure at two or only one side of the membrane surface (286a). When the lipid-protein interaction is predominately electrostatic (allowing only surface binding or partial penetration) both the enthalpy and the temperature of membrane lipid transition are affected (241, 286a). The effect of intrinsic proteins on the membrane lipid transition is generally limited to a reduction in the enthalpy of transition (241, 286a), with little alteration in the temperature of transition (51, 52, 277, 286a).

Many intrinsic proteins have been reported to be associated with a halo of phospholipids referred to as boundary lipids (165, 186, 355, 369) allowing distinction from the bulk phospholipids existing throughout the remainder of the membrane. Trauble and Overath (355) have suggested from their studies of Escherichia coli membranes, that intrinsic membrane proteins are surrounded by a boundary halo of 130 phospholipids and that the proteins affect another 500 phospholipids in such a fashion as to remove them from the bulk phase transition event. Gent and Ho (134) have also found evidence of

boundary lipids (protein perturbed) and demonstrated that above transition temperature there is a rapid exchange between these lipids and those of the bulk, but that very little exchange occurs below T_t where the bulk lipids are in the ordered state, and the boundary lipids are not. The presence of boundary lipids have also been documented in studies with proteins such as cytochrome C (239), sarcoplasmic reticulum ATPase (138, 165), and myelin proteolipid apoprotein (86, 87) in either systems containing synthetic lipids (86, 87, 134, 138, 165, 210, 239) or naturally occurring phospholipids (186).

Other substances present within or in contact with the cell membrane can also affect the temperature of membrane lipid phase transition. Divalent ions, such as Ca^{++} cause an increase in T_t (60, 358, 372) possibly through neutralization of negatively charged phospholipid head groups (358, 372). The presence of monovalent cations and anions (60) or changes in H^+ concentration (pH) (357, 372) also appear capable of altering bilayer fluidity. Cholesterol has also been shown to affect membrane phase transitions by lowering the T_t and lowering the the enthalpy of transition (286). Apparently cholesterol functions by allowing bilayers to exist over a wide range of temperatures in the semi-disordered-ordered state. At temperatures below the lipid transition temperature, the presence of cholesterol permits hydrocarbon chain fluidity while at temperatures above lipid phase transition temperature, cholesterol reduces phospholipid

hydrocarbon chain thermal motion (52, 286). As expected, membranes containing cholesterol as a normal component do not exhibit phase transitions (207).

H. Phase Transition Phenomenon in Biological Membrane Systems in Escherichia coli.

1. Mutants of Fatty Acid Metabolism. Silbert et al. (331, 332) were the first to isolate variants of Escherichia coli incapable of either synthesizing unsaturated fatty acids, (fabA$^-$ or fabB$^-$) (Figure 9) or of degrading the unsaturated fatty acids supplied exogenously (fad$^-$) (Figure 10). These mutants have allowed the study of membrane lipid phase transitions in isolated membrane fractions or in whole cells of Escherichia coli. Supplementing these Fad$^-$, Fab$^-$ double mutants, also called unsaturated fatty acid (Ufa$^-$) auxotrophs, with various unsaturated fatty acids does not alter the nature or the proportions of the species of phospholipids present (71, 116, 253, 281, 320) from that found in wild type cells (71). Thus, the lipids diphosphatidylglycerolphosphate, phosphatidylglycerol, and phosphatidylethanolamine comprise 7%, 15%, and 75% of phospholipids respectfully (71). Although various investigators have employed numerous unsaturated fatty acids in their studies, the majority of studies have included or used exclusively the unsaturated fatty acids cis-Δ^9-octadecenoic acid (oleic acid) and trans-Δ^9octadecenoic acid (elaidic acid). The basic reasons for utilizing these two unsaturated fatty acids center on the facts that: (1) growth of Ufa$^-$ auxotrophs on oleic acid results in membranes with fluidity characteristics and

Figure 9. Pathway of Fatty Acid Synthesis in Escherchia coli K-12
The synthesis of both saturated and unsaturated fatty acids in E. coli proceeds through a common pathway until synthesis of β-hydroxydecanoyl-ACP (acyl carrier protein) is complete. Once formed in the general pathway of fatty acid synthesis, β-hydroxydecanoyl ACP may serve as a substrate either for β-hydroxydecanoylthioester dehydrase (β, γ dehydrase) or for β-hydroxyacyl-ACP dehydrase (α, β dehydrase). The latter enzyme will result in the eventual synthesis of only saturated fatty acids. On the other hand β-hydroxydecanoylthioester dehydrase allows for the introduction of a cis double bonds at position 9,10 of the carbon chain. Following the action of either of these two enzymes, β-keto-acyl-ACP synthetase acts to condense malonyl-ACP with the β-keto-acyl-ACP. Apparently, β-keto-acylsynthetase has two sites of action, site I recognizes unsaturated fatty acids and site II recognizes saturated fatty acids. Except for the steps involving β-hydroxydecanoylthioester dehydrase and site I of β-keto-acyl-ACP synthetase the synthesis of saturated and unsaturated fatty acids proceed by the same cyclic pathway leading generally to C_{14}, C_{16}, or C_{18} saturated fatty acids and C_{16} and C_{18} unsaturated fatty acids. This pathway proceeds through (1) condensation by β-keto-acyl-ACP synthetase (2) reduction by β-keto-acyl-ACP reductase (3) dehydration by α, β-dehydrase and (4) reduction by enoyl-ACP reductase. Thus, mutations affecting unsaturated synthesis arise from alterations in the segment of the β-keto-acylACP synthetase gene coding for site I action (fabB$^-$) or in the gene coding for β-hyroxydecanoylthioester dehydrase (fabA$^-$).

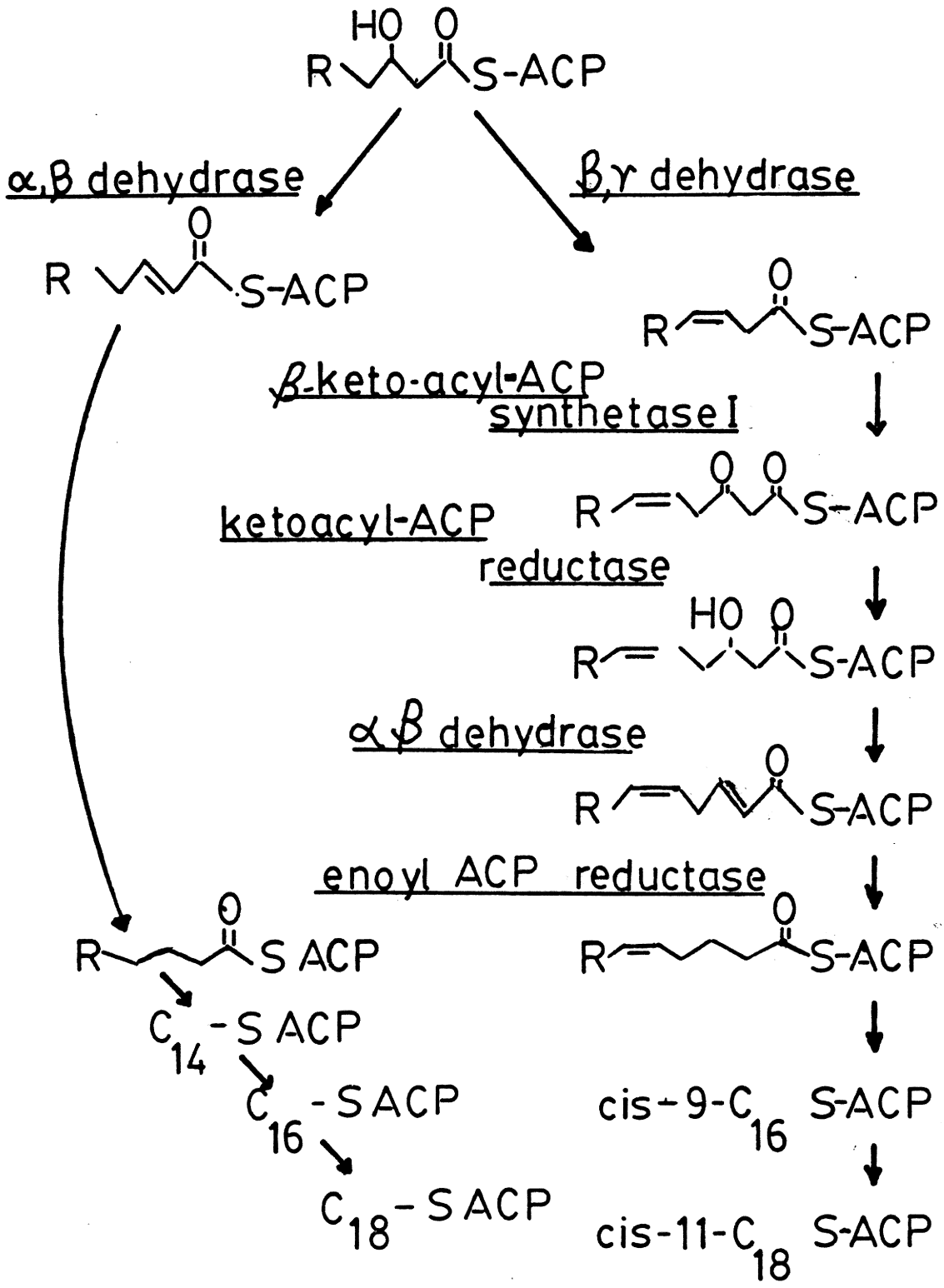
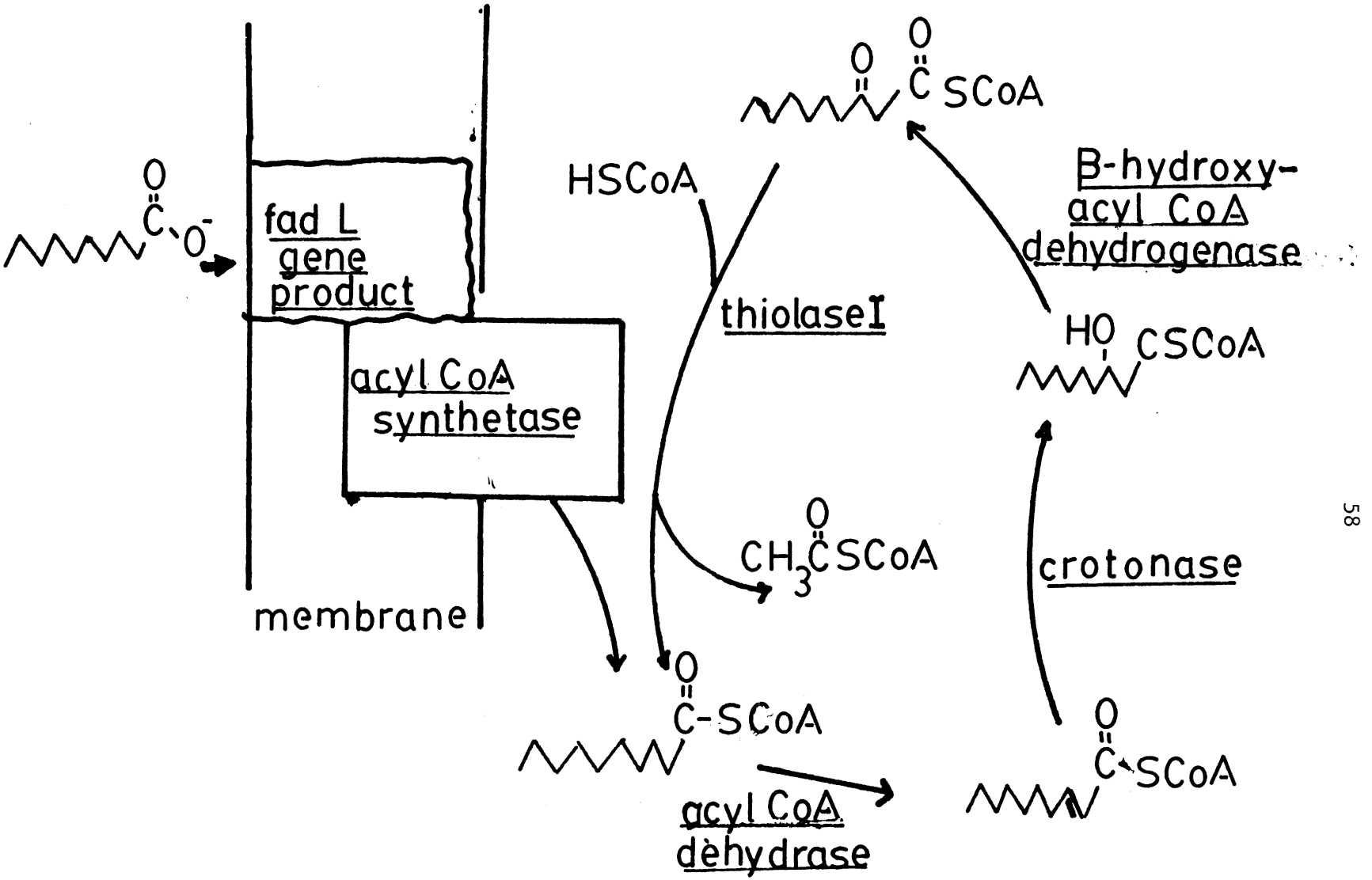

HO O
R S-ACP
α,β dehydrase
β,γ dehydrase
R S-ACP
R S-ACP
β-keto-acyl-ACP synthetase I
ketoacyl-ACP reductase
R S-ACP
HO O
R S-ACP
α,β dehydrase
R S-ACP
enoyl ACP reductase
R S ACP
R S-ACP
C14 - S ACP
C16 - S ACP
C18 - S ACP
cis-9-C16 S-ACP
cis-11-C18 S-ACP

Figure 10. Degradation of Fatty Acids in Escherichia coli K-12 (β-oxidation). Exogenously supplied fatty acids (exceeding 10 carbon units in length) are transported to cell interior by a specific transport system (fadL gene product) (256). As the fatty acid is transported across the membrane, acyl-CoA synthetase attaches Coenzyme A to the fatty acid forming an acyl-CoA thioester (256). Acyl-CoA is then passed through a cyclic pathway where 2 carbon units are removed with each turn in the pathway (196, 322). Mutations affecting some steps of the pathway have been identified and include those in genes coding for Acyl-CoA synthetase (fadD), an electron transport flavoprotein required for action of acyl CoA dehydrogenase (fadE), 3-hydroxyacyl-CoA dehydrogenase (fadB), and thiolase (fadA) (196, 322).


fad L
gene
product
acyl CoA
synthetase
membrane
HSCoA
thiolaseI
acyl CoA
dehydrase
crotonase
β-hydroxy-
acyl CoA
dehydrogenase

composition similar to that of wild type cells (277), (2) the presence of elaidic acid in cell membranes allows for phase transition induction within the normal temperature range of growth of Eschcerichia coli (129, 277), and (3) the obvious fact that oleate and elaidate differ in structure only by the orientation of the double bond at position 9,10 in the eighteen carbon structures.

2. Correlation of Physical with Physiological Changes During Phase Transition in Biological Membranes. Early studies with Ufa^- auxotrophs were initiated with the intention of correlating the well-established physical methods for determining phase transition with some physiologically measureable phenomenon which should have been affected by the phase transition event. The systems investigated in these early studies included cytoplasmic membrane transport systems, particularly the transport systems for β-galactosides (e.g. lactose) and β-glucosides (223, 319, 391).

Construction of Arrhenius plots from the data collected on the whole cell uptake of thio-β-D-methylgalactoside (TMG), an analog of lactose, by Ufa^- cells grown with different unsaturated fatty acids indicated that the temperature characteristics (slope of resulting line) varied considerably between the Ufa^- cells, depending upon the type of supplemented unsaturated fatty acid (319). Refining the procedure of Schairer and Overath (319), Wilson and Fox (391) found that for o-nitrophenol-β-D-galactoside (ONPG) hydrolysis (requiring a functional β-galactoside transport system) in cells grown on oleate or linoleate, an Arrhenius plot of the rate of hydrolysis versus temperature was bi-phasic, indicative of a phase transition (Figure 11).

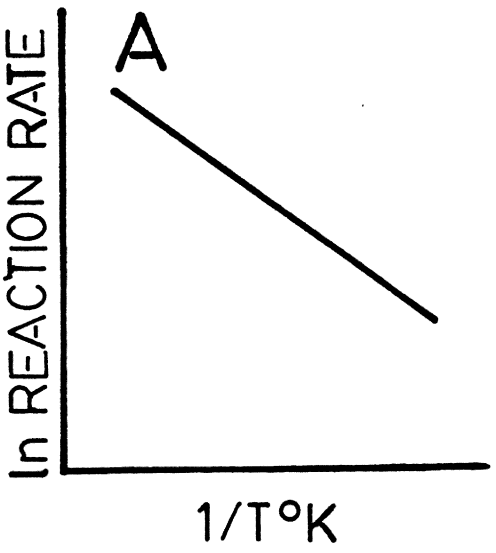
Figure 11. The Arrhenius Plot and Detection of Membrane Lipid Phase Transition. A plot of the rate of reaction as a function of the inverse of absolute temperature of reaction allows the estimation of the activation energy (Ea) of the reaction, the slope of the resulting line being equivalent to $-Ea/R$ (R is the gas constant).

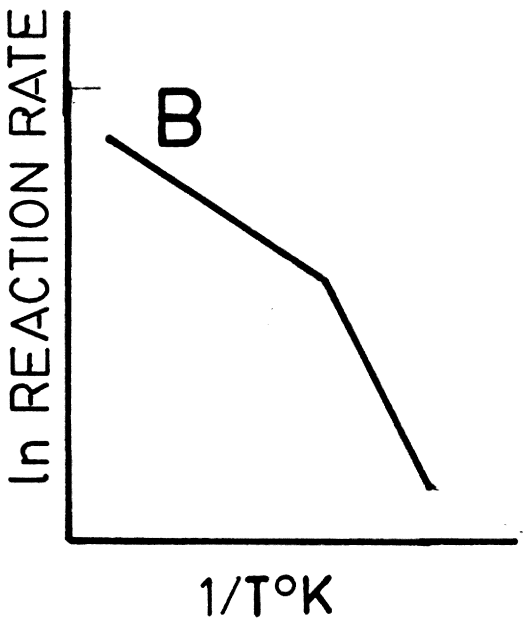
$$\frac{\ln K_{T_2}}{\ln K_{T_1}} = E_a \left[\frac{T_2 - T_1}{RT_2T_1} \right]$$

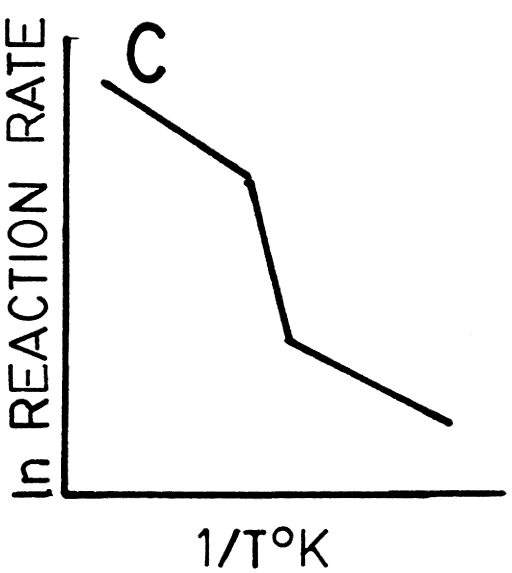
K_{T_2} = rate of reaction at temperature T_2

K_{T_1} = rate of reaction at temperature T_1

For most reactions, E_a is a constant value and a plot of lnK versus $1/T^{o}K$ yields a straight line (A). However, in reactions where the membrane serves as a supporting matrix for the enzyme reaction or transport function being investigated, membrane lipid phase transition may result in a discontinuity in the Arrhenius plot, the discontinuity occurring at the temperature of transition. Early investigations suggested only a single discontinuity (B) and generally these were reported to be indicative of a phase transiton of narrow ΔT. Latter reports suggested that two discontinuities were detectable (C) for some membrane-associated functions indicating a temperature for onset (T_h) and completion (T_l) of the phase transition. Information extracted from Williams and Williams (389).


A
ln REACTION RATE
1/T°K


B
ln REACTION RATE
1/T°K


C
ln REACTION RATE
1/T°K

Overath et al. (281) were the first to truly correlate changes in physiological phenomenon with the physically determined onset of phase transition. Bi-phasic Arrhenius plots for TMG efflux, respiration on glycerol, and growth were obtained when measurements for these functions were performed on Ufa$^-$ auxotrophs grown with either elaidate, oleate, or linoleate. The discontinuity in the bi-phasic Arrhenius plots correlated with the onset of the phase transition as measured by the force area isotherms of purified lipids isolated from these same cells (281). Esfahani et al. (115), using vesicles isolated from Ufa$^-$ cells after growth on various unsaturated fatty acids, found that the phase transition temperatures as identified by X-ray diffraction correlated with that determined by the discontinuity in the bi-phasic Arrhenius plot for proline uptake and succinic dehydrogenase activity (membrane bound respiratory enzyme). Schecter et al. (320, 321) also found that the discontinuity in Arrhenius plots for D-lactate-dependent, proline uptake by vesicles isolated from Ufa$^-$ auxotrophs correlated with phase transition in the same vesicles as determined by high angle X-ray diffraction patterns. Overath and Trauble (279) found that membrane phase transitions were detectable in whole cells using the fluorescent probe N-phenyl-1napthylamine (NPN) and that the transition temperature in whole cells as determined by NPN fluorescence as well as by Arrhenius plots for ONPG and methyl-α-galactoside efflux correlated with the phase transition in membrane vesicles determined by use of the fluorescent probes 8-anilinapthalene-1-sulfonate (ANS)

and NPN, and in phospholipids isolated from these cells as determined by 90^{o} light scattering and dilatometry.

Because physical determination of the membrane lipid phase transition correlated with rate changes in physiological phenomena, the alteration in rates of membrane-associated functions at the onset of the membrane lipid phase transition are collectively believed due to membrane phase transition. However, it is unresolved whether the the observed transitions affect rate constants or the effective molecular concentrations of the systems investigated (350). Letellier et al. (217, 349) attempted to resolve the issue by enumerating the lactose transport proteins in vesicles at temperatures above and below phase transition temperature by labeling the transport molecules with dansyl-galactoside. The investigation suggested a reduced level of binding to receptors below the transition temperature indicating a reduction in the number of available proteins. However, Overath et al. (280) found that dansyl-galactoside was transported by vesicles and then bound non-specifically internally as well as externally. Thus, the question remains unresolved.

Another problem which appeared in the study of phase transition in biological membranes was the apparent absence of a range of phase transition, ΔT, in biological membranes. Liposomes composed of a single phospholipid species demonstrate a relatively sharp single phase transition temperature (56, 166, 174, 293) whereas liposomes consisting of heterogeneous mixtures of phospholipids demonstrate a transition over a broad temperature range (58, 245, 292, 293, 328)

with T_h and T_l defining the extent of the range of transition (59, 129, 277). Initial investigations with Ufa$^-$ auxotrophs or their membrane fractions after growth with various exogenously supplied unsaturated fatty acids did not suggest, as would be expected, the presence of a broad transition. The discrepancy was resolved when Linden et al. (223) found that membrane fractions and whole cells of Ufa$^-$ auxotrophs did exhibit tri-phasic Arrhenius plots as measured with the fluorescent probe 2,2,6,6-tetramethylpiperidine-1-oxl (TEMPO) and, with more extensive measurements, β-galactoside and β-glucoside transport as well. Overath and Trauble (281) confirmed the findings of Linden et al., using 90^o light scattering, dilatometry, and air-water interface monolayer studies. Broad transitions were also obtained by Morrisett (253) in his study using NADH oxidase and D-lactate oxidase as physiological probes and fluorescent spin labels for physical measurement of the phase transition. Thilo et al. (350) also found tri-phasic Arrhenius plots for both N-phenyl-1-napthylamine fluorescence in whole cells and the transport functions forβ-glucosides and β-galactosides.

The studies suggesting a broad temperature range transition with T_h and T_l defining the extent of the transition is also supported by electron microscopy studies. Photographs of membranes above and below phase transition temperatures have shown that membranes cooled slowly to below transition temperature possess regions devoid of protein while other areas contain protein in quantities in excess of that observed in membranes above T_h, the upper end temperature of

transition (129, 216, 277, 320, 371, 372). Apparently the proteins partition between ordered and disordered domains as the temperature passes through T_h and T_l, their exclusion from the ordered regions (350) functioning as a probe for the phase transition. Membranes which are quickly frozen below T_l, the lower end of the phase transition, do not exhibit the segregation of proteins (371). Finally as noted with binary lipid vesicles (31, 144, 260), phase transition in the range between T_h and T_l for isolated membrane fractions result in increased permeability to electrolytes (e.g.,K^+) and non-electrolytes (e.g., erythritol) (144). The explanation for this observation is likely the same as found for binary systems where pores develop between phase interfaces resulting in increased permeability (175, 240, 286).

3. Membrane-Associated Functions Not Affected by Membrane Phase Transitions. Final clarification that membrane lipid phase transition does occur in whole cell membranes prompted investigations of the effect of membrane phase transition on membrane-associated functions other than transport and respiratory functions. Some membrane-associated systems proved to be unaffected by the phase transition. Mavis and Vagelos (244) concluded from their investigation of enzymes involved in phospholipid synthesis that although glycerol-3-phosphate acyltransferase is affected by membrane phase transition, the enzymes 1-acylglycerol-3-phosphate acyltransferase and sn-glycerol-3-phosphate dehydrogenase did not appear affected (244). Plate (294) found that a phase transition blocked the lethal action of a certain

antibacterial colicin, but that the colicin was not prevented from attaching to its receptor site under the same conditions. Some of the membrane-associated functions not affected by membrane phase transition are quite complex and likely involve many functions. Lofgren and Fox (225) reported that chemotaxis, but not mobility, is affected by altered membrane states. A very extensive investigation by Thilo and Veilmetter (352) was interpreted as indicating DNA replication and initiation of DNA synthesis occur normally in Ufa$^-$ cells when subjected to a membrane lipid phase transitions.

4. Assembly of Membrane Proteins into Gel-Phase Membranes.

The availability of the Ufa$^-$ auxotrophs has allowed for an extensive investigation of the influence of membrane fluidity on membrane protein assembly. For Gram-negative organisms, the synthesis of the cell envelope requires the assembly of cytoplasmic, periplasmic, and outer membrane components and thus a study on membrane protein assembly might require consideration of the physical status of both inner and outer membranes. Nikaido et al. (261) and Overath et al. (283) have found that both isolated outer and cytoplasmic membranes from Ufa$^-$ auxotrophs demonstrate phase transitions near the same temperature, but that the degree of transition in the outer membrane is reduced relative to that within the cytoplasmic membrane (313). A reduction in the length of the LPS in certain mutants generally results in a more ordered state in membrane lipids (313). Additionally, it was also suggested that the cytoplasmic membrane is more disordered than

the outer membrane above the transition temperature in both LPS-deficient and in wild type cells (253, 381).

Assembly of outer membrane proteins and periplasmic proteins is influenced by membrane lipid phase transition. Ito, et al. (180) reported that the onset of a membrane phase transition altered the rates of assembly of outer membrane proteins, particularly the periplasmic proteins. DiRienzo and Inouye (104) found that reduction of membrane fluidity results in the accumulation of the precursor to ompA gene product (pompA) in the cell envelope and inhibited the synthesis of outer membrane matrix proteins coded for by the ompC and ompF genes. Induction of the lactose operon in cells which have undergone a membrane phase transition (disordered to ordered) results in the synthesis of all proteins required for lactose utilization, although the transport complex (permease) was not assembled (360). Proteins inserted into the membrane are normally subject to extensive lateral diffusion, but this pheonomenon is not required for membrane protein insertion (282) as might be expected if protein insertion occurred at selected locations within the membrane. Additionally, further studies of the assembly of the lactose transport system suggested regional phase transitions can occur within the whole cell membrane leading to exclusion of newly synthesized proteins from the ordered regions. Tsukagoshi and Fox (361) found that Ufa$^-$ cells grown on elaidate and then suspended in oleate-containing medium under conditions for induction of lactose permease at a temperature between the transition point for membranes of oleate- and elaidate- grown

cells demonstrated only a single temperature of transition for β-galactoside transport (Arrhenius plot). This transition temperature correlated with the phase transition temperature of membranes from cells grown with oleate although in the same cell two transition temperatures were detected by physical techniques. It was suggested that newly formed transport system is associated with only newly synthesized oleate-containing phospholipids in the the disordered (oleate-containing) lipid phase and no newly synthesized protein associated with the elaidate-containing phospholipid in the ordered phase (360, 361). However, results from experiments conducted by Thilo and Overath (351) contradict the results of Tsukagoski and Fox, the more recent investigation indicating that only a single temperature of transition (physically or physiologically determined) occurs in cells grown first with elaidate and then suspended in oleate containing medium at temperatures between the phase transition temperature for membranes containing only one or the other unsaturated fatty acid. Thus, the transition temperature depends only on the molar ratio of the unsaturated fatty acids present in the phospholipids found in the cell membrane, and that mixing between new and old membrane component phospholipids readily occurs. Consequently newly synthesized protein readily diffused into areas of the membrane containing preformed lipid as long as the temperature was above transition the temperature of whole membrane.

In summary, it is apparent that membrane phospholipid phase transitions (as detected by physical techniques) are capable of

affecting some membrane-associated functions, particularly group translocations, active transport, and respiration functions. At this time it is not clear what happens at the molecular level; the phase transition could either reduce the frequency of the measured event or alter the number of available functional units participating in the event. Membrane protein assembly is also influenced by phase transition of membrane phospholipids but, again, the nature of the event is unclear and in dispute.

RATIONALE FOR EXPERIMENTAL APPROACH

A. Basis for Suspecting Role of Phospholipids in Conjugation

Exchange of membrane components between cells and vesicles (184, 185) and between cells which have entered into mating (93) suggest that membrane fusion can occur in cells of Escherichia coli. This process is apparently dependent on the presence of specific lipid species in specific ratios in the membrane bilayer as is demonstrated in studies with eukaryotic cells (42, 203) and artificial membrane systems (202, 203). Obtaining conditions which permit fusion may occur through either de novo synthesis of these specific phospholipids or alteration of the structure of existing lipids ( ). Collectively, these observations suggest an integral role for phospholipid metabolism and/or structure in the process of gene transmission by conjugation.

B. Requirements of an Experimental System Employed in the Investigation of the Role of Phospholipids in Conjugation.

Demonstration of this putative involvement of membrane phospholipids in conjugation requires an experimental system which permits an unequivical correlation between changes in membrane structure and/or composition with any changes in the ability to fulfill the requirements for successful completion of conjugation. To this end the development of the experimental system was governed by the following restrictions. (1) The system must allow for maintenance of cell viability within the strain(s) of Escherichia coli K-12 subject to changes in phospholipid metabolism. (2) Based on the premise that the degree of change in conjugation proficiency will be a reflection

of the extent of alteration in lipid structure and/or metabolism, the system must permit extensive changes in membrane phospholipid structure and/or composition i.e., a decrease in conjugation proficieny of less than 10-fold being difficult to unequivically establish as fact. (3) The system should allow for simple determination of any changes in conjugation proficiency and, simultaneously, permit analysis of which steps of conjugation are most affected by changes in membrane lipid structure and/or metabolism. Further discussion of these restrictions and their resolution follow below.

C. Characterization of Mutants of Lipid Metabolism and Identification of Those Strains Most Suitable for This Investigation.

In Escherichia coli the pathway of phospholipid synthesis lends itself to discussion in terms of a three stage process. Stage I consists of the procurment of phospholipid precursor compounds. This may be accomplished either by de novo synthesis or by transport of exogenously supplied serine (348), CTP precursors (218, 314) phosphate (30, 390), glycerol (155, 221) or fatty acids (196). Stage II of phospholipid synthesis may be considered to include the assembly of the phospholipid precursors compounds into an activated CDP-diglyceride (27, 28, 45, 48, 73, 74, 76, 77, 131, 190, 222, 268, 299 302, 303, 368) Stage III of phospholipid synthesis involves the conversion of CDP-diglyceride to the final form phospholipids found in the membrane (9, 49, 50, 71, 77, 106, 170, 189, 211, 298, 299).

The inability of Escherichia coli to transport phospholipids (184, 185) permits only isolation of conditional mutants (temperature or pH) at the level of stage II or III of phospholipid synthesis. To date, these mutants are found to exhibit phenotypes which do not readily permit their use in the investigation hereinbefore proposed. Expression of mutations in many steps of stage II phospholipid synthesis cause disruption of synthesis of macromolecular components (27, 132, 246) whereas expression of mutations in stage III of lipid synthesis generally result in negligible change in lipid composition (176, 297). Some mutations in stage III lipid synthesis do cause major alterations in lipid composition but the change is gradual following initiation of expression of the mutation and occurs simulataneous with disruption of synthesizing capacitiy for macromolecular components (299).

Opposed to the constraints encountered in the use of mutants of stage II and III in phospholipid synthesis, certain mutant strains of Escherichia coli in stage I of lipid synthesis permit the generation of membrane-associated phospholipids which are subject to extreme changes in the status of their physical state while maintaining the parameters which define normal conditions of growth for Escherchia coli (246, 278, 330, 331, 332). These strains are the unsaturated fatty acid auxotrophs previously described in the literature review as deficient in unsaturated fatty acid synthesis (fabA, or fabB) as well as fatty acid degradation (Fad^-) (278, 330, 331, 332).

Although the fabA, or B mutations in combination with the Fad^- phenotype allow for changes in lipid structure which are sufficient

to cause measurable changes in various physiological processes, there remains the question of the effect of the membrane lipid phase transition on cell viability, particularly since this factor has entered into the criticism of other investigations (75). Although problems may arise, this system is amenable allowing for adjustment of conditions (e.g. different unsaturated fatty acids, different temperatures, different medium, etc) which simultaneously allow maintenance of cell viability through a period of time where cells are subjected to the onset of membrane lipid phase transition.

D. Measurement of Conjugation Proficiency

In a further effort to limit the variables which might arise in this initial investigation of the role of phospholipids in conjugation, a mating system will be employed that minimizes the mating functions of the cell subject to the membrane phase transition event. In matings involving a donor of a relatively small plasmid (<3% of total DNA), the recipient cell need not expend energy (65, 89, 287) nor synthesize DNA (14), nor recombine the transferred fragment (63, 89) to ensure DNA transfer. Thus, the recipient cell shall be the cell subject to the membrane phase transition in this investigation. Furthermore, within minutes of mixing plasmid-donor and recipient cells measurable levels of plasmid transfer by conjugation occur (122); a factor which may prove important should maintenance of cell viability be limited over time. Use of a R-factor donor of the F-like plasmid group will allow easy detection of transfer in medium supplemented with appropriate antibiotics while simultaneously serving as a basis

for additional investigations employing those transfer factors for which the most information is available (F-type and F-like) (384). Finally a stable association of plasmid DNA within the recipient cells requires the establishment of the plasmid at the membrane attachment site (120) an additional function which may require a fluid membrane.

RESEARCH OBJECTIVES

The objectives of this research project are (1) development of a experimental system which allows the onset of membrane lipid phase transition in *fabB*$^-$, *fadE*$^-$ mutants of *Escherichia coli* K-12 while allowing for maintenance of cell viability (2) extend the above system to allow the investigation of the effects of a membrane lipid phase transition in recipient cells on conjugation (3) and to determine which steps, if any, are most affected by the onset of a membrane lipid phase transition in recipient cells.

MATERIALS AND METHODS

A. Bacterial Strains.

Table I lists the mutants used in this investigation. Variants carrying the R-factor, R100-1, were identified by their resistance to 50 ug/ml each of streptomycin, tetracycline, sulfonamide and chloramphenicol (94), sensitivity to bacteriophage f1, (224) and the ability to transfer both streptomycin and tetracycline resistance determinants by conjugation. Variants lacking thymidine kinase (Tdk^-) were identified by the method of Hiraga *et al.* (168, 177) and by the inability to incorporate ^3H-thymidine into the trichloroacetic acid (5%) insoluble fraction. Low level thymidine-requiring variants were obtained from trimethoprim-resistant (Thy^-) mutants (340), arising spontaneously following growth in the presence of 2 ug thymidine/ml (226, 340) contained in MSC-1% glucose (see below).

It was found that strain K1059 and its derivatives that are auxotrophic for unsaturated fatty acids quickly lost viability upon refrigeration of stock cultures containing growth factors. Extension of refrigerated stock shelf-life was obtained by growing cells in oleate-containing medium followed by suspension in minimal salts medium prior to refrigeration. Stock cultures, so prepared, stored for up to a month without risk of either significant loss of viability or selection of Fab^+, or Fad^+ revertants.

Table I
Bacterial Strains

Strains	Relevant Genotype/Phenotype	Source
Ymel	F^-, λ^+	B. Bachmann
χ59	F^-, *thr*$^-$, *leu*$^-$, *thi*$^-$	R. Curtiss III
OT100	F^-, *met*$^-$, λ^r, *lac-44*	lambda resistant variant of 1308; this investigation
JF107	F^-, *lac*$^-$	J.O. Falkinham III
OT135	R100-1 /*met*$^-$, λ^r	lambda resistant variant of 1886; this investigation
OT155	R100-1/ *met*$^-$, λ^r, Thy$^-$	trimethoprim selection with OT135; this investigation
OT196	F^-, *fabB*$^-$, *fadE*$^-$, *lacI*$^-$ λ^+ $T6^r$	selection of T6 resistance this investigation
OT197	F^-, *fabB*$^-$, *fadE*$^-$ *lacI*$^-$, λ^+ $T6^r$, *trp*::Tn10	transduction of OT196 with P1 lysate of NK5151 this investigation
OT198	R100-1/ *fabB*$^-$, *fadE*$^-$ *lacI*$^-$, λ^+	transconjugant of OT155 x K1059; this investigation
OT199	$F^-\lambda^+$, *fabB*$^-$, *fadE*$^-$, *lacI*$^-$, Tdk$^-$, $T6^r$	transduction of OT197 with P1 lysate of KY895; this investigation
χ289	F^-, wild type	R. Curtiss III
KY895	F^-, *tdk*$^-$1 , *ilv*$^-$	S. Hiraga
K1059	F^-,*fabB*$^-$, *fadE*$^-$, λ^+	P. Overath
χ1308	F^- *met*$^-$,	R. Curtiss III
χ1886	R100-1/ *met*$^-$	R. Curtiss III
NK5151	*trp*::Tn 10	N. Kleckner

B. Media.

The semi-defined medium used for growth consisted of minimal salts (MS) medium (88), to which was added 0.2% vitamin-free Casamino acids (Difco, Detroit, Mich.) and is abbreviated as MSC. The carbohydrate supplement is indicated by a suffix to MSC indicating the concentration and the type of carbohydrate added: e.g., 0.5% glycerol (w/v); MSC-0.5% glycerol, 1% glycerol (w/v); MSC-1% glycerol, or 1.0% glucose (w/v); MSC-1%-glucose.

The unsaturated fatty acid requirement of Escherichia coli K-12 strain K1059 and its variants was met by the addition to MSC-0.5% glycerol of either oleate (cis-Δ^9-$C_{18:1}$; octadecenoic acid, Sigma) or elaidate (trans-Δ^9-$C_{18:1}$; octadecenoic acid, Sigma Co.) at 0.02% (w/v) (319). Fatty acids were solubilized with the addition of 0.2% (v/v) Brij 35 (Sigma) (319). Oleate-supplemented MSC-0.5% glycerol is abbreviated as MSCBO and elaidate-supplemented MSC-0.5% glycerol is abbreviated as MSCBE. Absence or presence of either Casamino acids, oleate, elaidate, or Brij 35 (in the concentrations indicated above) in various other media is indicated in the medium abbreviation by the presence or absence of the letters "C", "O", "E", or "B" respectfully.

Either Luria Broth (250) or LB (250) complex medium each supplemented with 2.5 mM $CaCl_2$ was used for growth of cells prior to infection with T6 or P1 bacteriophage in preparation of phage lysates. Lambda broth (250) was used for growth of cells prior to infection with lambda phage (382). When used for growth of unsaturated fatty acid

auxotrophs, complex media were supplemented with 0.2% (v/v) Brij 35 and 0.02% oleate (w/v). Preparation of solid medium was permitted by the addition of agar (Difco) at concentrations of either 1.5% (w/v) or 1.2% (w/v) when required for preparation of phage lysates.

Frequency of reversion to Fad^+ phenotype for strain K1059 and its variants was determined on MS agar plates supplemented with 0.1% oleate and 1.0 % Brij 35. The Fab^+ phenotype was monitored on MSC-1% glucose.

C. Genetic Techniques.

Stock lysates of bacteriophage P1L4 and lambda were prepared following the methods of Curtiss (94) using the Escherichia coli strains χ289 and χ59 respectfully. Strain χ289 grown on either Luria Broth or LB medium (2.5mM Ca^{++}) (250) was the host for T6 bacteriophage. For ^3H-thymidine-labeled plasmid transfer experiments, T6 bacteriophage lysates were prepared on strain χ289 grown on Luria Broth (2.5 mM Ca^{++}) and the phage precipitated with 5% polyethylene glycol, centrifuged, and suspended in Hershey Chase medium (163) to approximately 2×10^{11} plaque forming units (pfu)/ml.

In preparation for selection of T6-resistant mutants of strain K1059, cells were first grown on LB medium containing 0.2% Brij 35 and 0.02% oleate prior to suspension in medium lacking oleate and Brij 35. The cell suspension was then cross-streaked with T6 bacteriophage at 4×10^9 pfu/ml on LB plates containing oleate and Brij 35. Colonies arising in region of lysis and clearing were streaked for purity three times on appropriately supplemented Luria broth and isolates then

tested for resistance to T6 by cross streaking (as above) and the inability to support plaque formation by T6.

To prepare strain K1059 and its variants for transduction, cells were grown to exponential phase in LB containing 2.5 mM $CaCl_2$, 0.02% oleate, and 0.2% Brij 35, centrifuged, and concentrated 10-fold in 1% tryptone. To 0.5 ml of the concentrated cell suspension was added 0.5 ml of P1 phage (at a multiplicity of infection of between 1 and 10) which had previously been suspended in adsorption medium consisting 0.015M $CaCl_2$ and 0.03M Mg_2SO_4. The mixture was then incubated 30 minutes at 37 C and putative transductants were centrifuged, suspended in MS prior to plating. The Trp::Tn10 variant (OT197) was obtained following transduction by plating on MSBO-1% glucose agar containing 10 ug tetracycline/ml and 40 ug tryptophan/ml. The Tdk^-, Trp^+ variant (OT199) of strain OT190 was obtained following transduction by plating on MSBO-1% glucose agar with no supplements. Phage techniques employing other strains were the same as above with the exception that oleate and Brij 35 were deleted from the medium.

A derivative (OT198) of strain K1059 carrying the R-factor, R100-1, was obtained through conjugation with χ1886 in the absence of fatty acid and detergent, followed by selection of cells resistant to 50 ug streptomycin/ml on MSBO-1% glucose agar medium. Presence of the R-factor was confirmed by demonstration of resistance to the antibiotics characteristic of R100-1 and transfer of the same.

D. Growth Requirements of Unsaturated Fatty Acid Auxotrophs.

Cultures of strain K1059 or its variants were always inoculated from overnight cultures only after the fresh medium was prewarmed to growth temperature (39.5 C). Cultures of 10 to 20 ml in 250 ml Erlyenmeyer flasks were aerated with reciprocal rotary motion (40 to 50 cycles/min) in a New Brunswick shaker bath. Cell number was determined by viable count on MSCBO agar plates following dilution of cells in MS prewarmed to growth temperature. Cell mass was monitored by optical density measurements following 1:10 dilutions in MS at 470 nm on a Hitachi 102 digital spectrophotometer. The dilution step reduced the turbidity of media at temperatures below 38 C due to the presence of elaidate.

Experimental design often required a change of culture conditions. Normally a simple procedure, this proved a problem with mutant strain K1059 due to the extreme temperature sensitivity of this K-12 variant. A method allowing high recovery of viable cells was developed as follows: cells growing at 39.5 C were rapidly collected on 0.22 um cellulose nitrate filters (Millipore) of 45 mm diameter, washed with two volumes MS prewarmed to 39.5 C, and aseptically transferred on the filter to a 250 ml Erylenmeyer flask containing either MS or another growth medium (varying with experiment) prewarmed to desired temperature. Cells were completely (99.9%) removed from filter in 30 to 60 seconds (as determined by viable count and spectrophotometry) by rotating the flask at 40 to 50 rotations/min. From start to finish the procedure took less than 3 minutes.

E. Mating Experiments.

Recipient cells were grown to early exponential phase (1 to 2 x 10^8 cells/ml) in either MSCBO or MSCBE at 39.5 C and collected by filtration. Cells on membrane filters were suspended in MS prewarmed to 39.5 C as described above. Small volumes of MS-suspended cells were then shifted to mating temperature (within 2 to 7 minutes of initiation of filtering) 1 minute prior to the addition of donor cells. Donor cells grown without shaking in 5 ml of MSC-1% glycerol at the mating temperature were added to the recipients in quantities allowing for donor to recipient ratios of either 1:1 or 1:10. Mating was terminated by agitation at high speed on a vortex mixer (Scientific Industries Inc.) for 10 seconds followed by rapid dilution in MS prewarmed to mating temperature. Recipient cells which had acquired R100-1 plasmid during mating were then selected at 39.5 C on MS agar containing 50 ug streptomycin/ml, 1% glucose, 0.02% oleate, and 0.2% Brij 35. A portion of recipient cell suspension not utilized in matings was used to measure the frequency of Fab^+ and Fad^+ revertants. For matings terminated by selective lysis of donor by bacteriophage T6, a 0.1 ml volume of the mating mixture was mixed with 1 ml of LB containing 5 mM KCN and 1 x 10^9 T6 bacteriophage pfu/ml for 5 minutes at 37 C prior to plating on selective medium.

F. Reversibility of Mating Inhibition Caused by Temperature Shift.

A 30-ml volume of recipient cells was grown in MSCBO or MSCBE at 39.5 C, filtered and suspended by the above procedure into 27 ml of MS prewarmed to growth temperature. Aliquots of 9 ml from this

suspension were then transferred to incubators at either 36.5 C, 33.5 C or left at 39.5 C. One minute after the shift, 1 ml of MSC-1% glycerol at the temperature of recipient cell suspension was added to simulate the addition of donor cells. At 10 and 30 minutes following the temperature shift, recipient cells were returned to 39.5 C and allowed to mate 30 minutes with MSC-1% glycerol-grown donor cells (39.5 C) at a donor recipient ratio of 1:10. Transconjugants were selected as previously described.

G. Stability of R100-1 Plasmid In Strain K1059.

The variant of strain K1059 (OT198) carrying the plasmid R100-1 was grown in MSCBE at 39.5 C until reaching early exponential phase. The culture was then divided into two aliquots; one remained at 39.5 C in MSCBE and the other, following filtering and suspension in MSC-1% glycerol (39.5 C) was shifted to 33.5 C for a period of 30 minutes and then back to to 39.5C immediatedly following a second filtering and resuspension in MSCBO prewarmed to 33.5 C. This series of steps was designed to mimic the mating procedure. Throughout the duration of the temperature shift to 33.5 C, samples were collected for measurement of colony forming ability on MSCBO-1% glycerol agar and MSCBO-1% glycerol agar plates supplemented either individually with streptomycin at 50 ug/ml and tetracycline at 10 ug/ml or with both streptomycin and tetracycline together at 50 ug/ml each and 10 ug/ml respectfully. Plates were incubated at 39.5 C.

H. Mating Pair Measurement.

Matings were initiated as described in "Mating Experiments" above. Fifteen minutes after the mixing of cells, pair formation was determined. Where mating pairs were measured by the method of Skurray (335) as modified by Eckerson and Reynard (110), the mixture was diluted in prewarmed (mating temperature) MS and plated on lactose-tetrazolium (335, 250) agar containing 0.02% oleate and 0.2% Brij 35. Measurement of pairs by particle counter was done by the method of Falkinham and Curtiss (119) using a ZB Coulter Counter® equipped with a 30-um orifice.

I. Release of Free Lambda Phage Upon Onset of Temperature Shifts in Strain K1059.

Strain K1059 cells were grown in either MSCBO or MSCBE at 39.5C and then filtered and suspended in MSC-0.5% glycerol to mimic mating conditions as described above (Stability of R100-1). Cell number was determined by measuring viable count on MSCBO-0.5% glycerol agar. The the release of free lambda bacteriophage into the medium was determined by mixing culture dilutions in MS (39.5 C) with 2.5 ml of melted (45 C) Lambda top agar (250) containing 0.1 ml of exponential phase culture of 59 grown in Lambda Broth. The mixture was gently agitated on a vortex mixer and poured over prewarmed (37 C) Lambda agar plates (250) followed by incubation at 37C for 8 to 12 hours prior to counting plaques.

J. Transfer of ^{3}H-Thymidine Labeled R100-1 DNA.

The K1059 Tdk^{-} variant (strain OT199) was grown and prepared for mating as above except that the MS used for final suspension of cells

prior to mating contained 400 ug thymidine/ml and 200 ug deoxyguanosine per ml. (MS tdr/gdr). Thy$^-$ donor cells (0T155) were grown in MSC-1% glycerol supplemented with 200ug deoxyguanosine/ml (394) and 3H-thymidine (20Ci/mmol; Amersham Corp.) at a final concentration of 2 ug/ml in 1.5 ml volumes. A 0.5 ml volume of properly prepared (as above) recipient cell suspension at mating temperature (39.5 C or 33.5 C) was added to 0.5 ml of donor cells to initiate the 30 minute mating period.

Matings were terminated at either 0 or 30 minutes by transferring 0.2 ml of mating mixture to 1 ml of Hershey-Chase buffer (163) containing 2 x 10^{11} UV-irradiated (800 ergs/mm^2) T6 bacteriophage (383), 5mM KCN (prepared day of experiment), 100 ug thymidine/ml and 40 ug tryptophan/ml. Following a 15-minute incubation to allow selective lysis of donor cells, labeled donor DNA and other cellular debris was solubilized by the method of Hiraga et al. (169) using DNase I (Bovine Pancreas Type I; Sigma Co.), RNase A (Bovine Pancreas Type I-AS; Sigma Co.) Streptomyces griseus protease (Sigma Co.) and Brij 58 (Sigma Co.).

Putative transconjugants were mixed with 2 x 10^8 carrier cells/ml (UV-irradiated; 4000 ergs/mm^2 in MS), followed by two washes in MS containing 0.1M $Na_4P_2O_7$ (Mg++ chelator, blocking DNase). Recipient cell DNA was then precipitated at 4 C for at least one hour with an equal volume of 10% TCA containing 200 ug thymidine/ml. After incubation, the suspension was filtered over GL/C filters (Gelman Co.)

previously wetted with 0.1M $Na_4P_2O_7$ and washed with 10 ml each of 0.1M $Na_4P_2O_7$ and of 0.3M NH_2COOH (pH 7.8) and 95% alcohol by the method of Wang et al. (375).

Filters were collected and radioactivity determined on a Beckmann 3100 scintillation spectrophotometer in 10 ml of a counting fluid consisting of PPO-POPOP primary and secondary fluors in toluene (Econofluor, New England Nuclear).

RESULTS

A. Survival of Strain K1059 Cells Following a Temperature Shift to 30 C.

Unsaturated fatty acid (Ufa^-) auxotrophs when grown with certain unsaturated fatty acids are subject to a temperature-induced membrane phase transition within the same temperature range normally used for growth of wild type cells of Escherichia coli (75, 189, 277, 281). Upon the initiation of a temperature-induced membrane phase transition in these cells there is an immediated cessation in the increase of cell number and cell mass. Thus, these two measurements have served as a simple barometer for the onset of change in the physical status of membrane lipid (75, 189, 281, 352). Demonstration of this same behavior in elaidate-grown cells of the Ufa^- auxotroph, strain K1059, was the anticipated result when following the procedure described by Thilo and Veilmetter (352). However, the use of viable count as opposed to particle counter for the determination of cell number led to the discovery that such a temperature shift of elaidate-grown cells caused a loss in colony forming ability within the population of cells.

Oleate-grown cells of strain K1059, exhibited very little change in growth rate, represented by viable cell enumeration and optical density measurement, upon a temperature shift from 39.5 C to 30 C (Figure 12), paralleling the the results of Thilo and Veilmetter (352). In contrast, elaidate-grown cells of strain K1059 exhibited an immediate cessation in the increase in cell mass (Figure 13) upon the

Figure 12. The Effect of a Temperature Shift to 30 C on Viability of Oleate-Grown Cells of Escherichia coli Strain K1059. A culture of strain K1059 in MSCBO at 39.5 C was divided into two fractions upon reaching the mid-logrithmic phase of growth (indicated by the zero mark); one fraction remained at 39.5 C (■ , □) and the other fraction was shifted to 30 C (● , ○). Cell number was monitored by viable count on MSCBO agar (closed symbols) and cell mass was estimated by determination of optical density at 470 nm (open symbols) following 1:10 dilutions of culture in MS (open symbols). Result depicted is representative of three separate experiments.

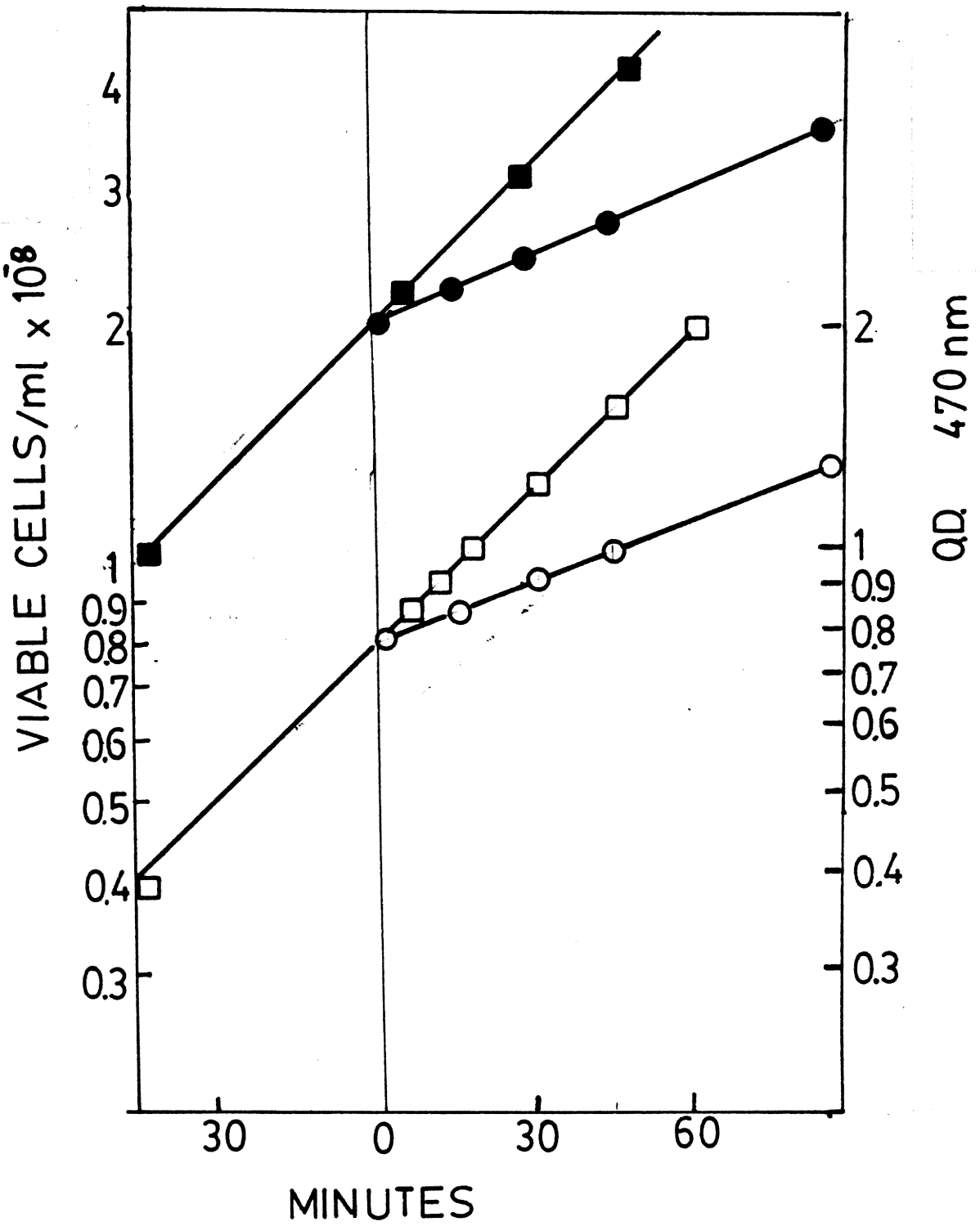
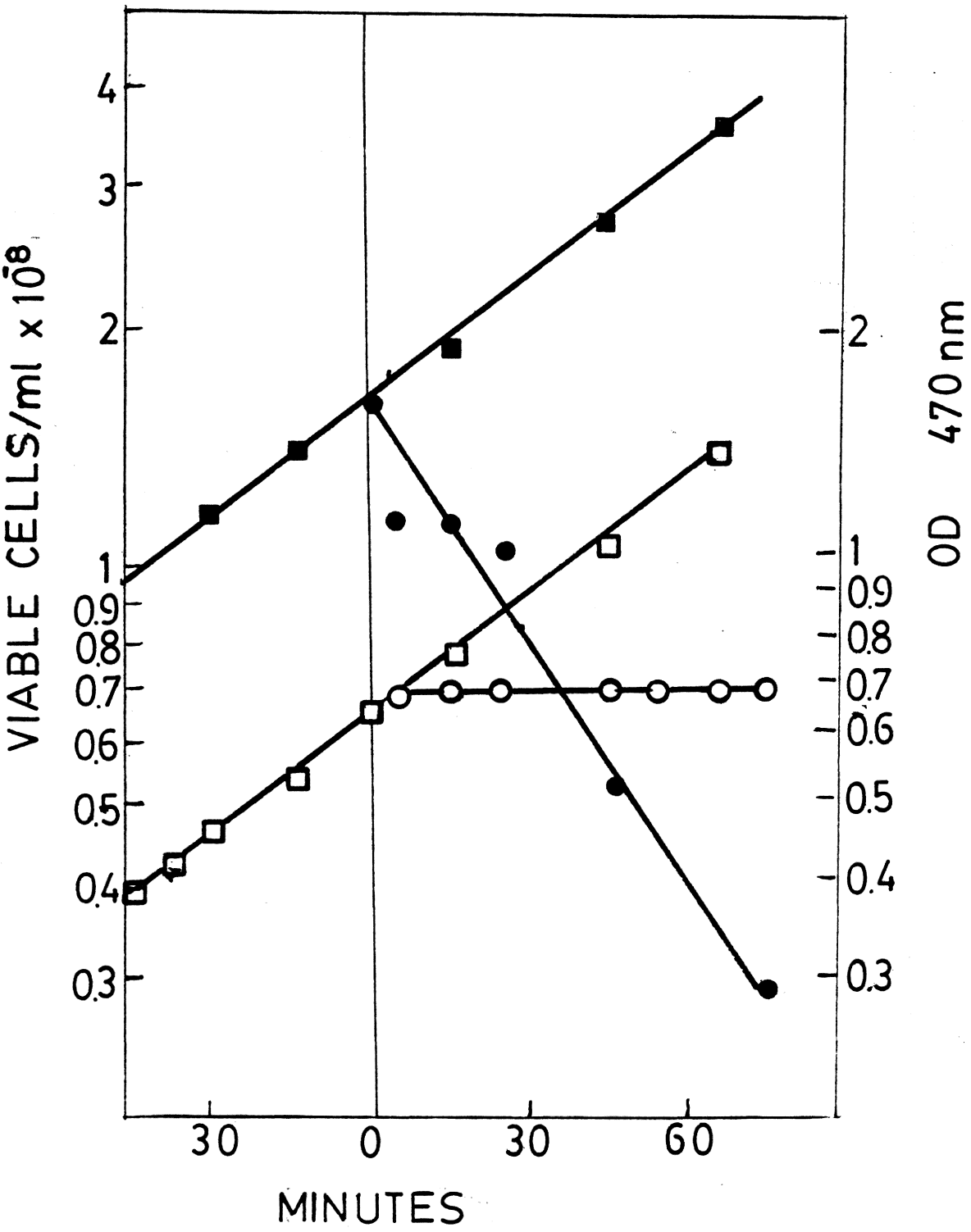

VIABLE CELLS/ml x 10⁸
4
3
2
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
2
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
O.D. 470 nm
30
0
30
60
MINUTES

Figure 13. The Effect of a Temperature Shift to 30 C on Viability of Elaidate-Grown Cells of Escherichia coli Strain K1059. A culture of strain K1059 in MSCBE at 39.5 C was divided into two fractions upon reaching the mid-logrithmic phase of growth (indicated by the zero minute mark); one fraction remained at 39.5 C (■ , □) and the other fraction was shifted to 30 C (● , ○). Cell number was monitored by viable count on MSCBO agar (closed symbols) and cell mass was estimated by determination of optical density at 470 nm (open symbols) following 1:10 dilution of culture in MS for cells at 39.5 C and 30 C. Result depicted is representitive of 3 separate experiments.


VIABLE CELLS/ml x 10⁸
4
3
2
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
OD 470 nm
2
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
30
0
30
60
MINUTES

initiation of the temperature shift to 30 C. Unexpected, was the fact that the temperature shift to 30 C caused a very rapid decrease in viable cell number (Figure 13).

Because the forementioned investigation had not indicated a loss of cell viability in in elaidate-grown cells of Ufa$^-$ mutants when shifted to temperatures of 30 C or below, the possibility was considered that the observed loss of viability in elaidate-grown cells was an artifact of the specific technique used in either the growth of the cells or the plating of the cells for viable count. However, variation in the procedures used for growth or for plating failed to alter the response of elaidate-grown Ufa$^-$ mutant cells to the temperature shift to 30 C. Prewarming pipettes, dilution medium, and plating medium to either 30 C or 39.5 C prior to viable count determination at 39.5 C failed to reduce the loss of colony forming ability with the onset of a temperature shift. Varying the suspension medium used for the dilution step in viable count between buffered saline with gelatin (88), MS, MSCBE, MSCBO (30 C and 39.5 C) also failed to reduce the temperature-induced killing. Different plating media (MSCBO, MSBO1% glycerol, MSCBO 1%-glucose, and Luria broth supplemented with 0.02% oleate and 0.2% Brij 35) all yielded identical results relative to viability of elaidate-grown cells of strain K1059 at 30 C.

The possiblity that the basal salts medium, MS, might affect the elaidate-grown Ufa$^-$ cells response to the temperature shift was also explored. The use of Cohen-Rickenberg mineral salts medium (11),

supplemented with 0.5% glycerol, 0.3% casamino acids (vitamin-free), 0.2% Brij 35, and 0.02% elaidate, as described by Overath et al. (281) did not result in alteration of the Ufa$^-$ cells response to a temperature shift. Although the ionic strength of support medium has been shown to influence the temperature of membrane phase transition in in vitro systems [high ionic strength lowering the temperature of transition (129)] the use of a low ionic strength mineral salts medium, Medium 63 (250), also failed to prevent the onset of cell death for elaidate-grown cells of strain K1059 at 30 C (Figure 14).

It is apparent that the use of a particle counter for the determination of cell number by other investigators resulted in the failure to detect cell death of elaidate-grown cells of the Ufa$^-$ mutant at 30 C. Overcoming the problem of cell death is crucial to the investigation into the effect of a membrane phase transition on conjugation because measurement of conjugation requires the viability of transconjugants. Thus, assuming that cell death is not an absolute consequence of a membrane phase transition, an attempt was initiated to identify the factor(s) that were responsible for causing loss of cell viability in elaidate-grown cells of strain K1059 when exposed to 30 C.

First considered was the possiblity that some factor was being generated during cell growth (e.g., a breakdown product of the detergent) which was functioning as a lethal agent when cells were shifted to 30 C due to alterations in cell envelope permeability. However, as illustrated in Figure 15, elaidate-grown cells of strain

Figure 14. The Effect of Growth in Low Ionic Strength Medium Prior to and During a Temperature Shift to 30 C on Viability of Elaidate-Grown Cells of Strain K1059. A culture of strain K1059 in Medium 63 supplemented with 0.02% elaidate, 0.2% Brij 35, 0.5% glycerol, and 0.2% casamino acids (vitamin-free) was divided into two fractions upon reaching the mid-logrithmic phase of growth (indicated by zero minute mark); one fraction remained at 39.5 C and the second fraction was shifted to 30 C. Cell number was determined by viable count on Medium 63 agar supplemented as above (oleate replacing elaidate) for cells at 39.5 C (■) and 30 C (O). Result depicted is representative of two experiments.

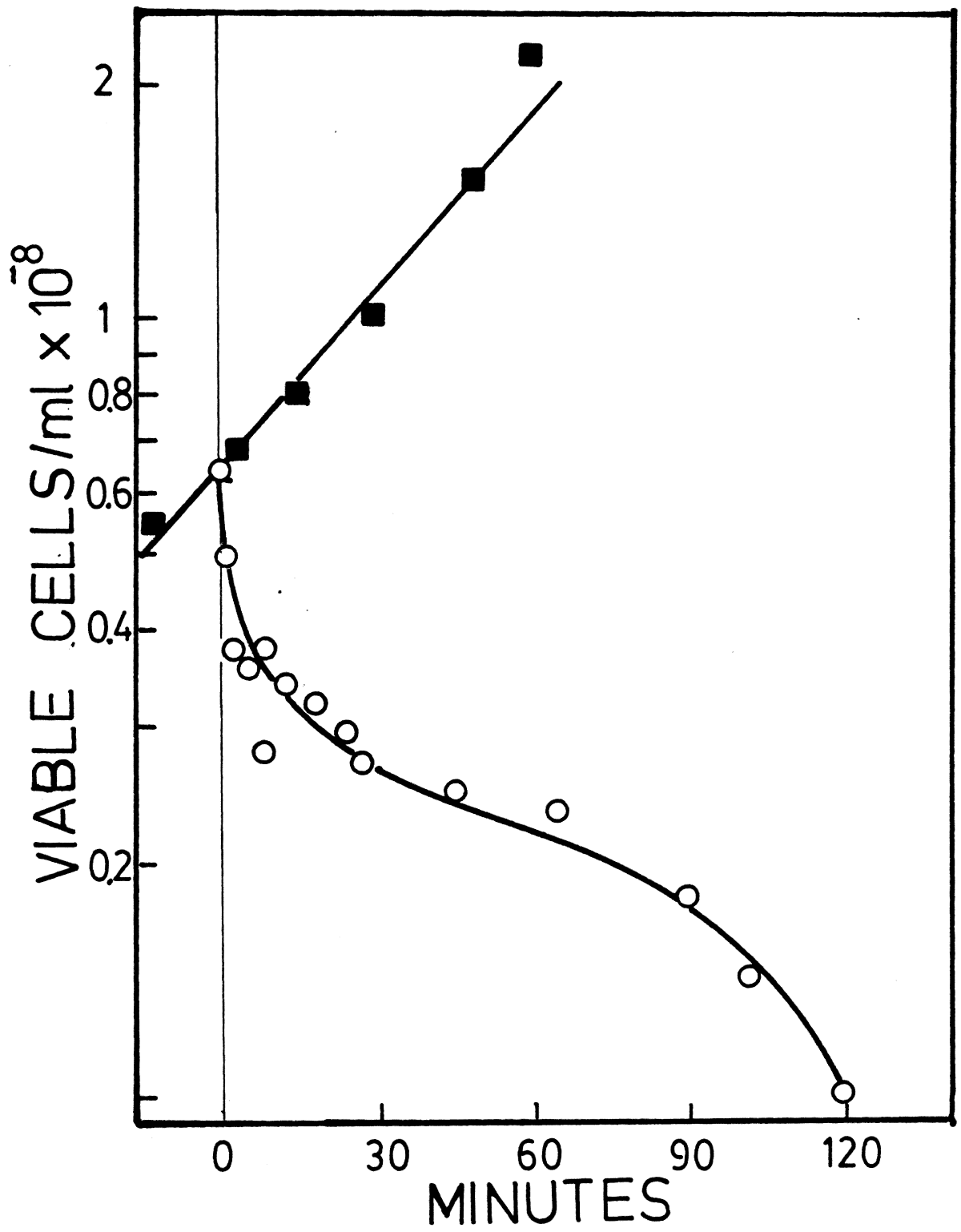
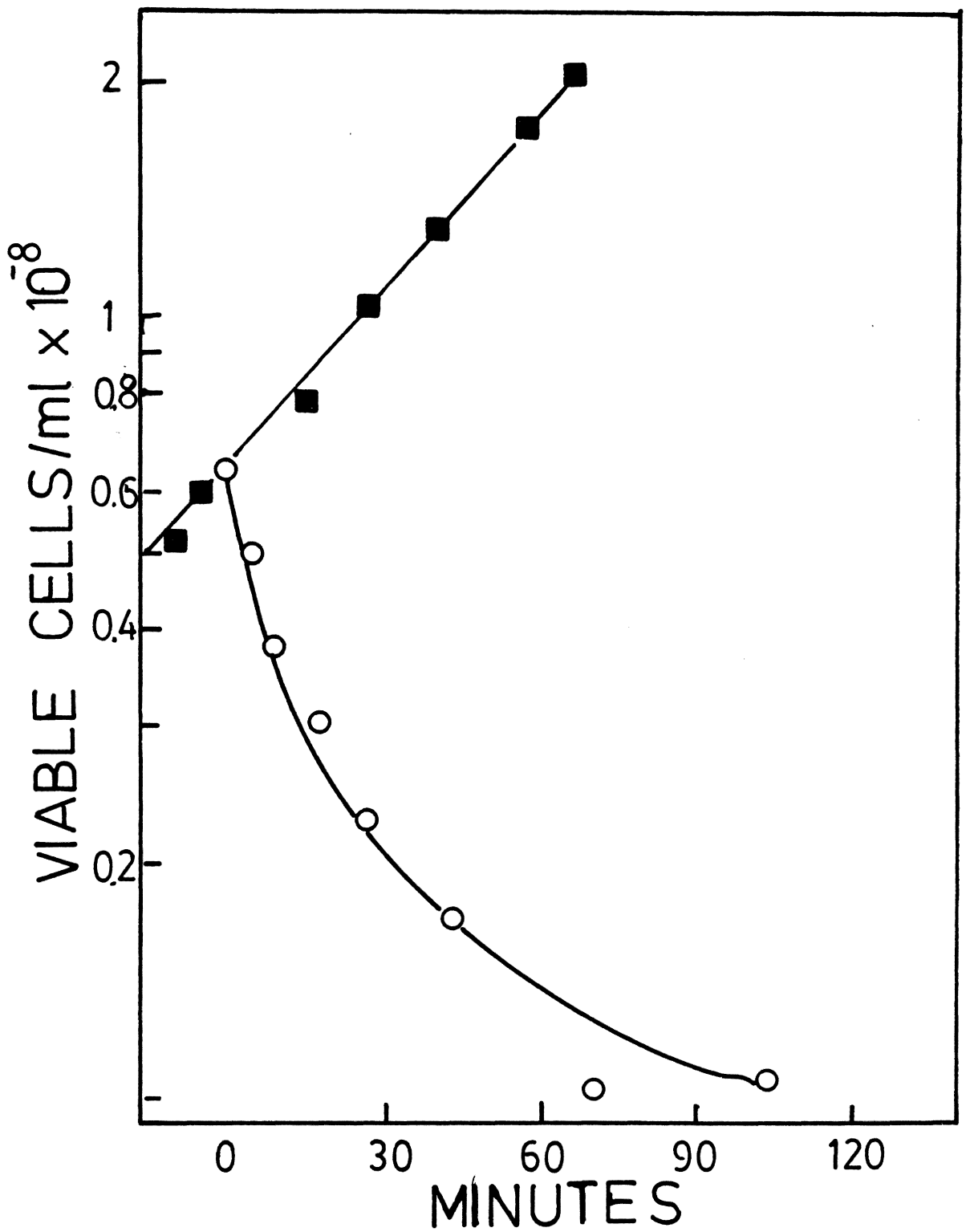

VIABLE CELLS/ml x 10⁻⁸
2
1
0.8
0.6
0.4
0.2
0
30
60
90
120
MINUTES

Figure 15. The Effect of Suspension in Fresh MSCBE Prior to a Temperature Shift to 30 C on Viability of Elaidate-Grown Cells of Strain K1059. Cells of strain K1059 in MSCBE at 39.5 C were collected and divided into two fractions upon reaching the mid-logrithmic phase of growth (indicated by the zero minute mark); one fraction remaind at 39.5 C in MSCBE and the second fraction was filtered and suspended in fresh MSCBE (39.5 C) prior to a temperature shift to 30 C. Cell number was monitored on MSCBO agar for cells at 39.5 C (■) and 30.0 C (O). Result depicted is representative of two experiments.

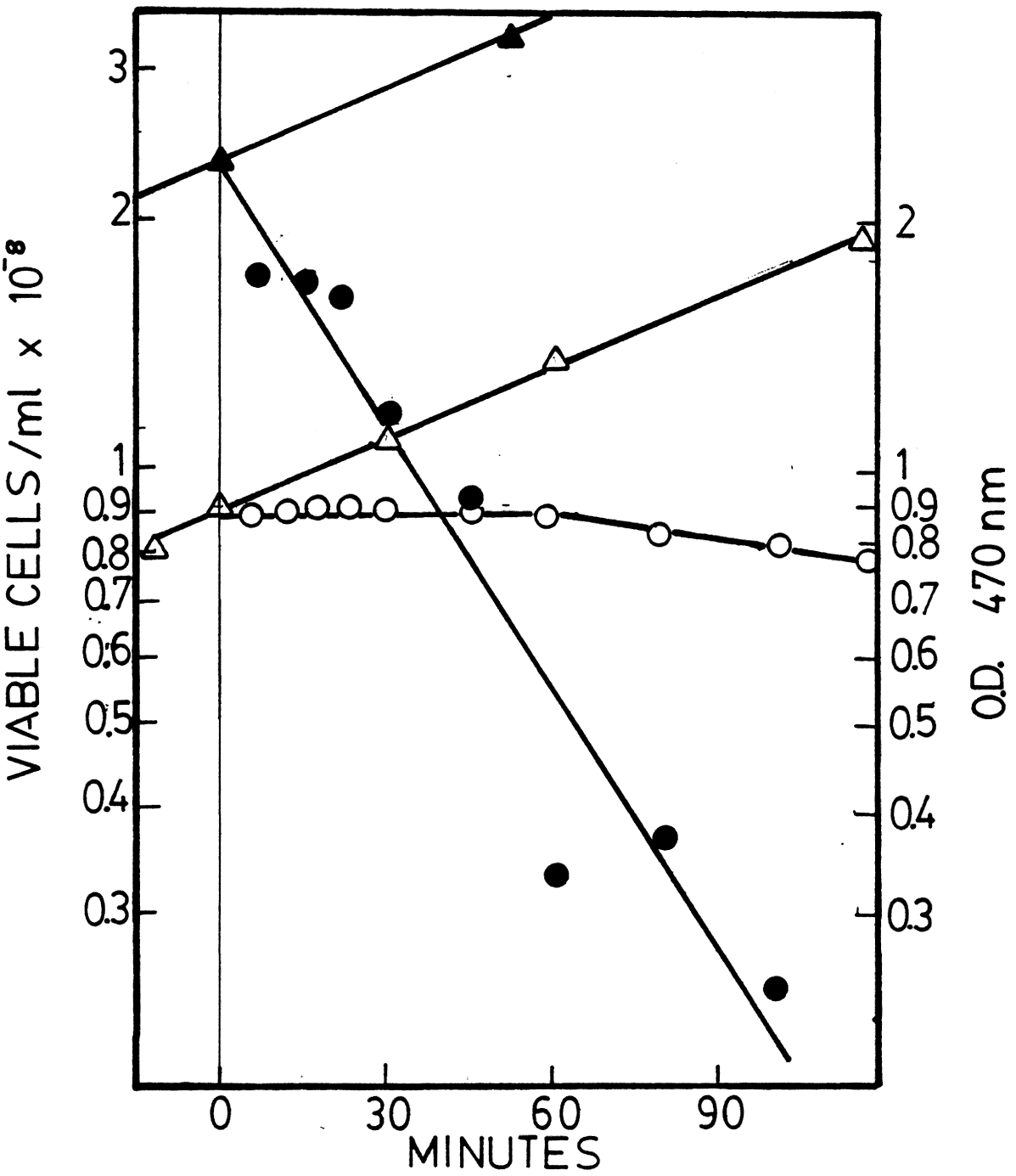

VIABLE CELLS/ml x 10^{-8}
2
1
0.8
0.6
0.4
0.2
0
30
60
90
120
MINUTES

K1059 suspended in fresh medium just prior to a temperature shift to 30 C are still subject to rapid cell death, thus voiding this hypothesis.

The possibility existed that some component present in the medium during growth was the factor responsible for causing cell death. A temperature shift experiment of elaidate-grown cells of strain K1059 in defined medium (MS supplemented with 0.2% Brij 35, 0.02% elaidate 1.0% glycerol and 40 ug/ml each of methionine and threonine) indicated that cell death was as rapid in the defined medium at 30 C as in MSBCE-0.5% glycerol despite the fact that the growth rate at 39.5 C was only half that of cells in MSBCE-0.5% glycerol (Figure 16).

It was next necessary to determine if the other factors required for growth (glycerol or elaidate) or the fatty acid solubilizing agent (Brij 35) was responsible for cell death at 30 C in elaidate-grown cells. In order to investigate the above possiblities, a method was required which allowed removal of these substances from the growth medium without subjecting the cells to low temperatures or to extended periods without fatty acids. It was found that the cells could be collected on sterile membrane filters (0.22 um pore dia, Millipore 45 mm dia.) and aseptically transferred to fresh medium without loss in viable cell number as determined by viable count. The entire procedure including a two volume wash with MS (39.5 C) required less than 2 minutes, thus minimizing any effects due to the absence of fatty acids from the medium. Using this procedure prior to the onset of a temperature shift, it was found that viability of cells suspended in minimal salts

Figure 16. The Consequence of Growth in Minimal Medium Prior to and During a Temperature Shift to 30 C on Viability Elaidate-Grown Cells of Strain K1059. A culture of strain K1059 in MS supplemented with 0.02% oleate, 0.2% Brij 35, 1.0% glycerol, and 40 ug/ml each of threonine and methionine was divided into two fractions upon reaching the mid-logrithmic phase of growth (indicated by the zero minute mark); one fraction remained at 39.5 C (▲, △) and the second fraction was shifted to a 30 C (●, ○). Cell number was determined by viable count on MSCBO agar (closed symbols) and cell mass was estimated by optical density measurements at 470 nm (open symbols) following 1:10 dilutions in MS. Result depicted is representative of two experiments.


VIABLE CELLS/ml x 10^{-8}
O.D. 470 nm
MINUTES
0
30
60
90
3
2
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3

lacking a carbon source could be maintained for at least 45 minutes at 30 C as is demonstrated in Figure 17. This observation suggested that some supplement to MS was the factor responsible for causing rapid cell death upon initiation of the temperature shift to 30 C.

When elaidate-grown cells of strain K1059 were suspended in MS containing Brij 35 (0.2%) prior to the temperature shift, they survived at 30 C for periods in excess of 30 minutes without loss in cell viability (Figure 18). However, the presence of 0.5% glycerol (Figure 18) or 0.5% glycerol and Casamino acids (Figure 19) was found to result in cell death at 30 C for elaidate-grown cells of strain K1059. This result suggested that cell death occurred upon the onset of the membrane phase transition in the presence of a metabolizable carbon source and may not be affected by the presence or absence of the unsaturated fatty acid, elaidate. This hypothesis was confirmed (Figures 20, 21) when the ability of elaidate-grown cells of strain K1059 to survive at 30 C in medium containing various carbon sources and elaidate is compared to viability of cells suspended in MS. Although the differences are relatively modest, the greater availability of metabolizable carbon source the greater the rate of cell death upon initiation of the temperature shift. In order, cell death occurred with increased rate in the presence of 0.05% glycerol, 0.1% glycerol, or 0.1% glycerol and 0.2% casamino acids (Figure 20). Cells suspended in MS containing 0.5% glucose exhibited a loss of cell viability at a rate similar to that observed for cells at 30 C in MS-1% glycerol plus casamino acids (Figure 21).

Figure 17. The Consequence of Suspension in MS Prior to a Temperature Shift to 30 C on Viability of Elaidate-Grown Cells of Strain K1059. A culture of strain K1059 in MSCBE at 39.5 C was divided into three fractions upon reaching the mid-logrithmic phase of growth (indicated by the zero minute mark); one of the three fractions was filtered, washed, and suspended in MS (39.5 C) immediately prior to a temperature shift to 30 C (◆, ◇) while the two remaining fractions remained in MSCBE and were either shifted to 30 C (●, ○) or left at 39.5 C (■, □). Viable cell number was determined on MSCBO agar (closed symbols) and cell mass was estimated by optical density measurements at 470 nm (open symbols) following 1:10 dilution in prewarmed MS (39.5 C or 30 C with respect to temperature of culture) Result depicted is representative of three separate experiments.

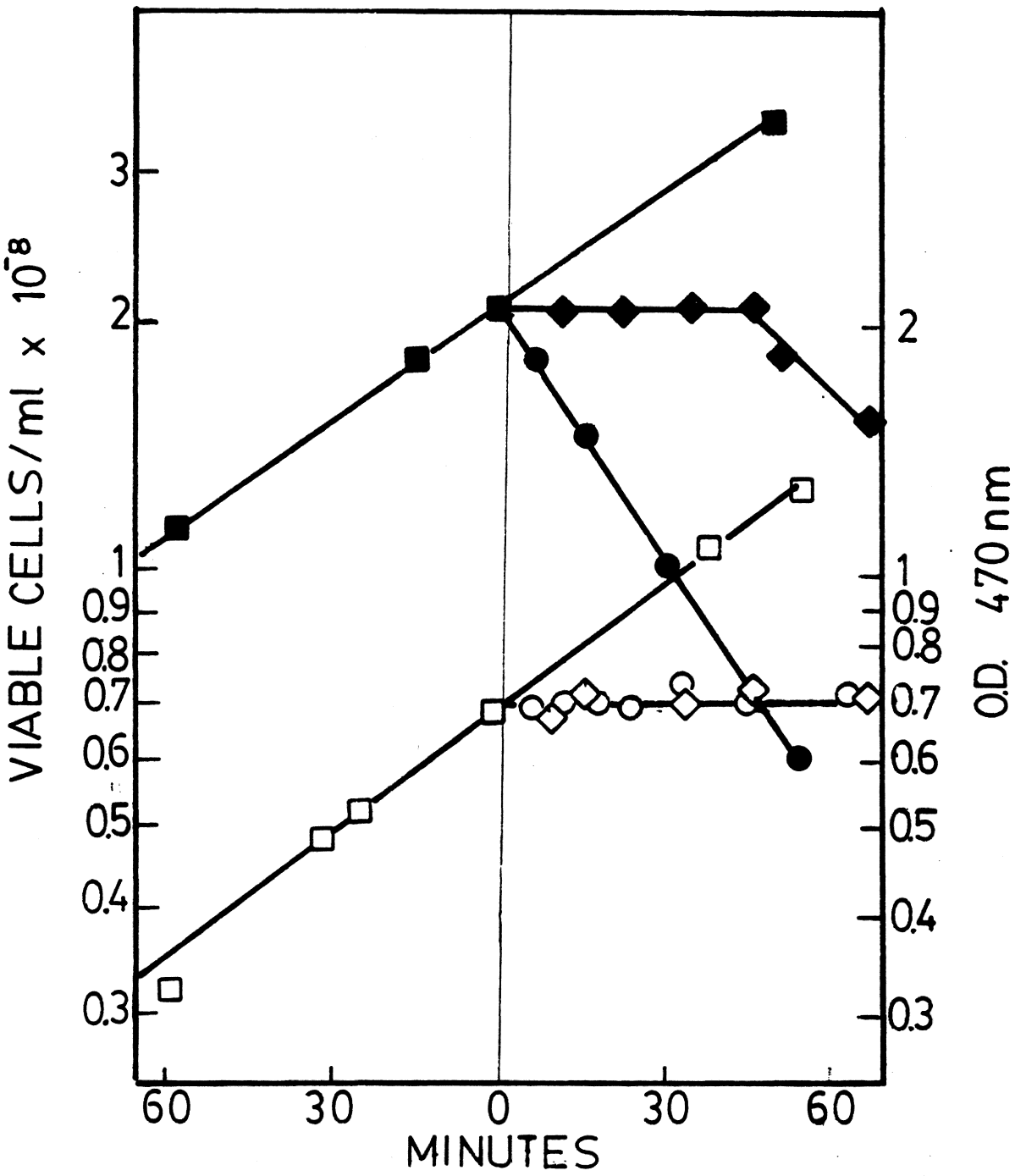

VIABLE CELLS/ml x 10^{-8}
3
2
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
2
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
O.D. 470nm
60
30
0
30
60
MINUTES

Figure 18. The Effect of the Absence of Glycerol on Viability at 30 C of Elaidate-Grown Cells of Strain K1059. A culture of strain K1059 in MSCBE at 39.5 C was divided into three fractions upon reaching the mid-logrithmic phase of growth (indicated by the zero minute mark). One fraction remained at 39.5 C in MSCBE (■ , □) while the remaining two fractions were filtered and suspended in MS (39.5 C) with 0.5% glycerol (● , ○) or without glycerol (◆ , ◇) prior to a shift to 30 C. Viable cell number was determined by colony forming ability on MSCBO agar (closed symbols) and cell mass was estimated by optical density measurements (470 nm) following a 1:10 dilution in MS prewarmed to culture temperatures (open figures).

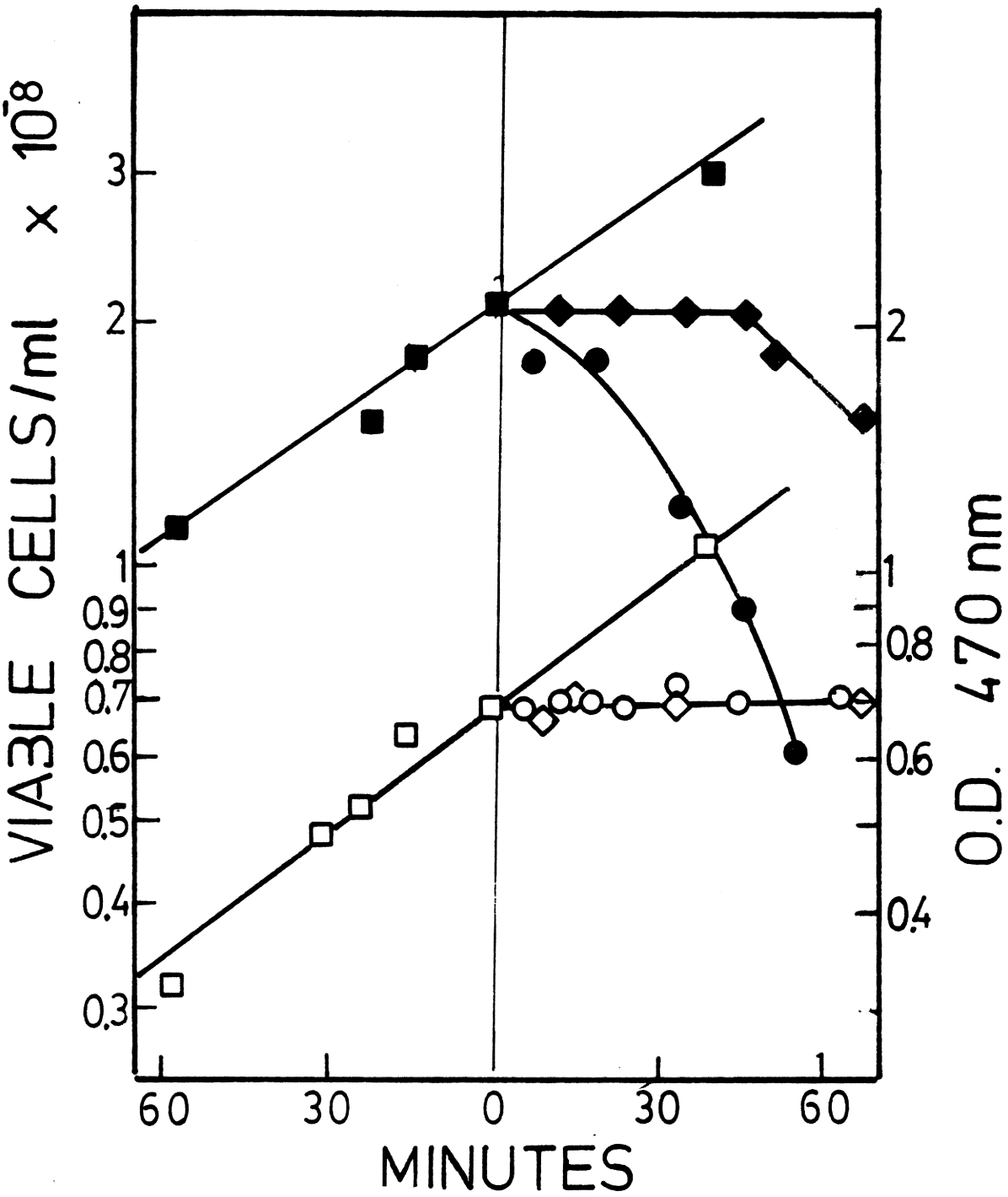

VIABLE CELLS/ml x 10⁸
3
2
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
2
1
0.8
0.6
0.4
O.D. 470 nm
60
30
0
30
60
MINUTES

Figure 19. The Effect of the Presence of Multiple Growth Factors on Viability at 30 C of Elaidate-Grown Cells of Strain K1059. A culture of strain K1059 in MSCBE at 39.5 C was divided into five fractions upon reaching the mid-logrithmic phase of growth (as indicated by the zero minute mark). With the exception of a control remaining at 39.5 C in MSCBE, cells were suspended in MS (39.5 C) supplemented in various ways and then shifted 30 C. Cell number was determined by viable count on MSCBO agar for cells in MSCBE at 39.5 C (□), and cells at 30 C in MS (△) or MS supplemented with either 0.5% glycerol (□) 1% glycerol (○) or 0.5% glycerol and 0.2% casamino acids (◇). The depicted result is representative of two experiments.

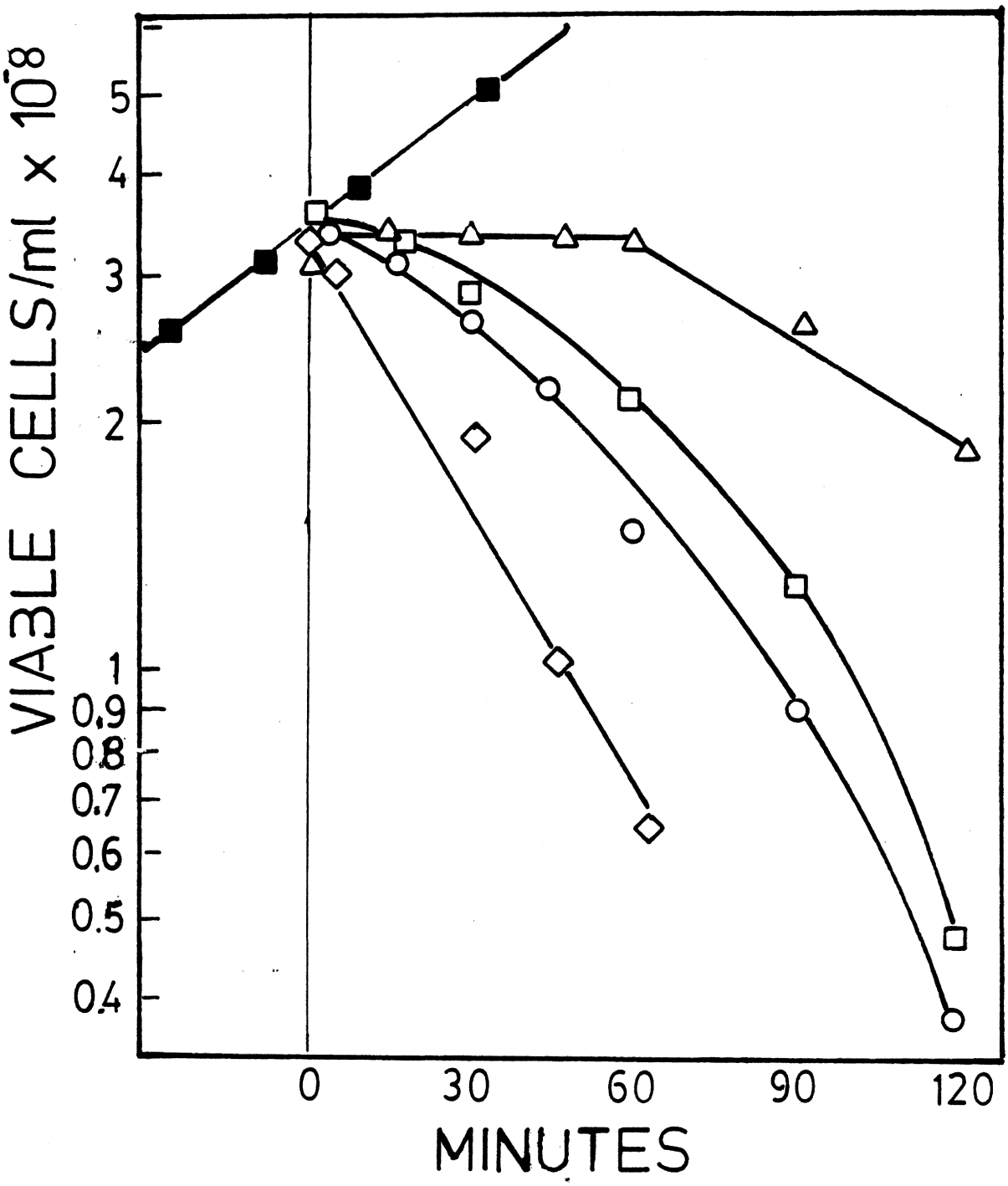

VIABLE CELLS/ml x 10⁸
5
4
3
2
1
0.9
0.8
0.7
0.6
0.5
0.4
0
30
60
90
120
MINUTES

Figure 20. The Effect of Elaidate on Viability at 30 C of Elaidate-Grown Cells of Strain K1059. A culture of strain K1059 in MSCBE at 39.5 C was divided into four fractions upon reaching the midlogrithmic phase of growth (indicated by the zero minute mark). One fraction remained in MSCBE at 39.5 C (■) and the remaining fractions were filtered and suspended in MS containing 0.02% elaidate. One of these fractions was shifted to 30 C (●) and the other two fractions were shifted to 30 C after further supplementation with either 0.5% glycerol (◆) or 0.5% glycerol and 0.2% casamino acids (▲). Cell number was determined by viable count on MSCBO agar (filled symbols) and cell mass was estimated by optical density measurments at 470 nm (open symbols) following 1:10 dilution in MS. The depicted result is representative of two experiments.

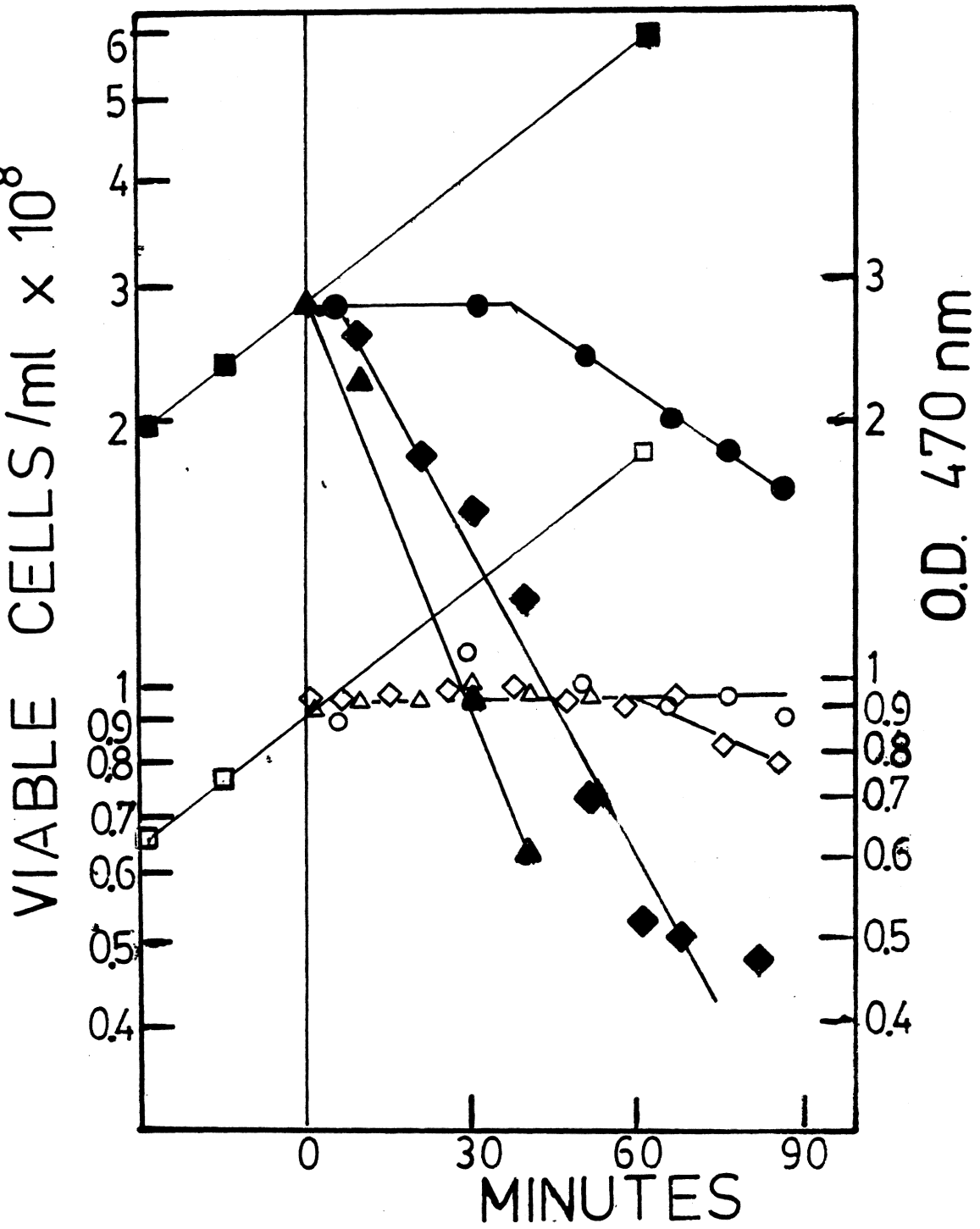
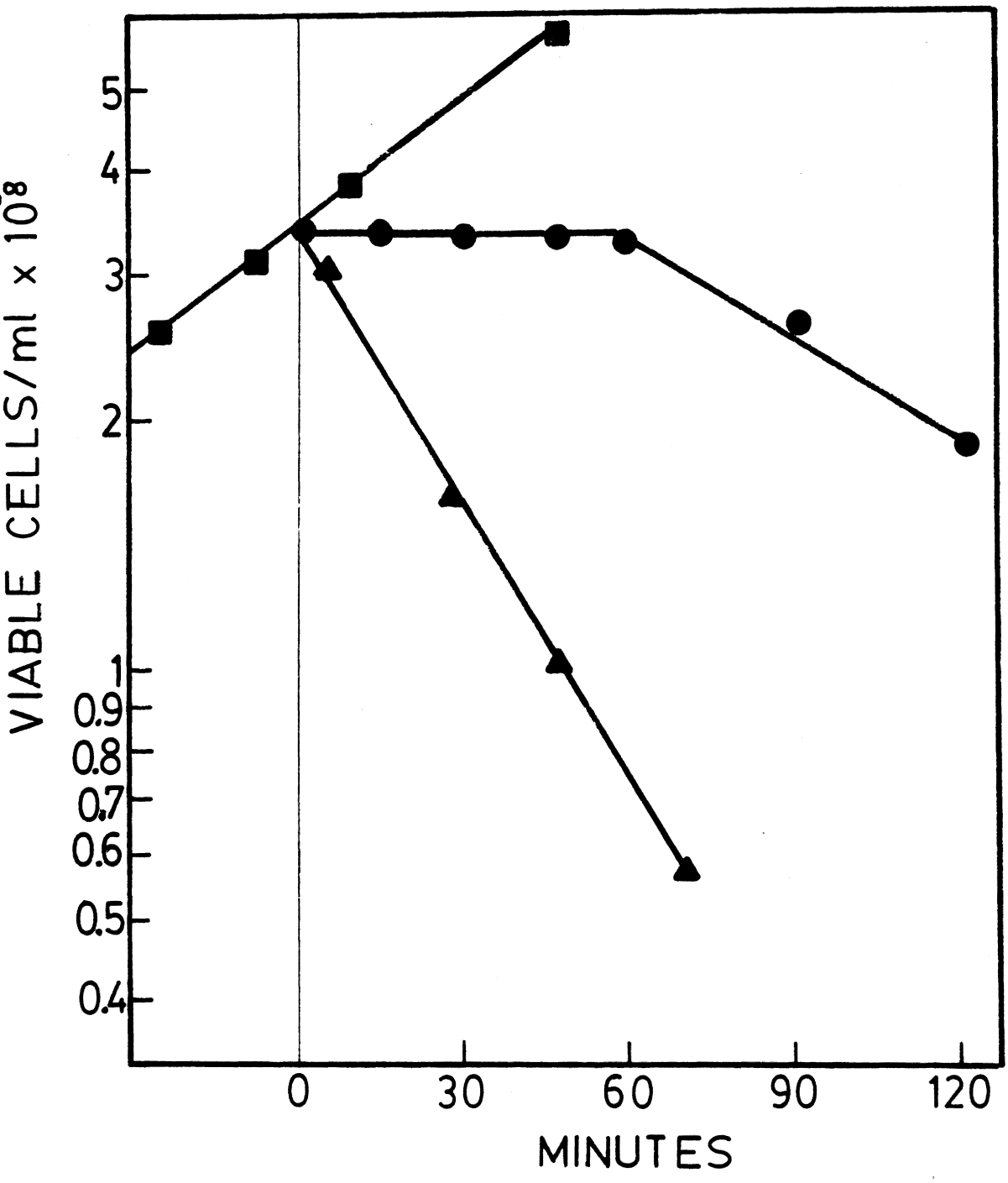

VIABLE CELLS/ml x 10^{-8}
O.D. 470 nm
MINUTES
0
30
60
90
6
5
4
3
2
1
0.9
0.8
0.7
0.6
0.5
0.4

Figure 21. The Effect of the Presence of Glucose on Cell Viability at 30 C of Elaidate-Grown Cells of Strain K1059. A culture of strain K1059 at 39.5 C in MSCBE was divided into three fractions upon reaching the mid-logrithmic phase of growth (indicated by the zero minute mark); one fraction remained at 39.5 C in MSCBE (■) and the two remaining fractions were shifted to 30 C immediately following filtering and suspension in either MS (●) or MS containing 0.5% glucose (▲). Colony forming abilty on MSCBO agar was used to estimate cell number. Result depicted is representative of duplicate experiments.

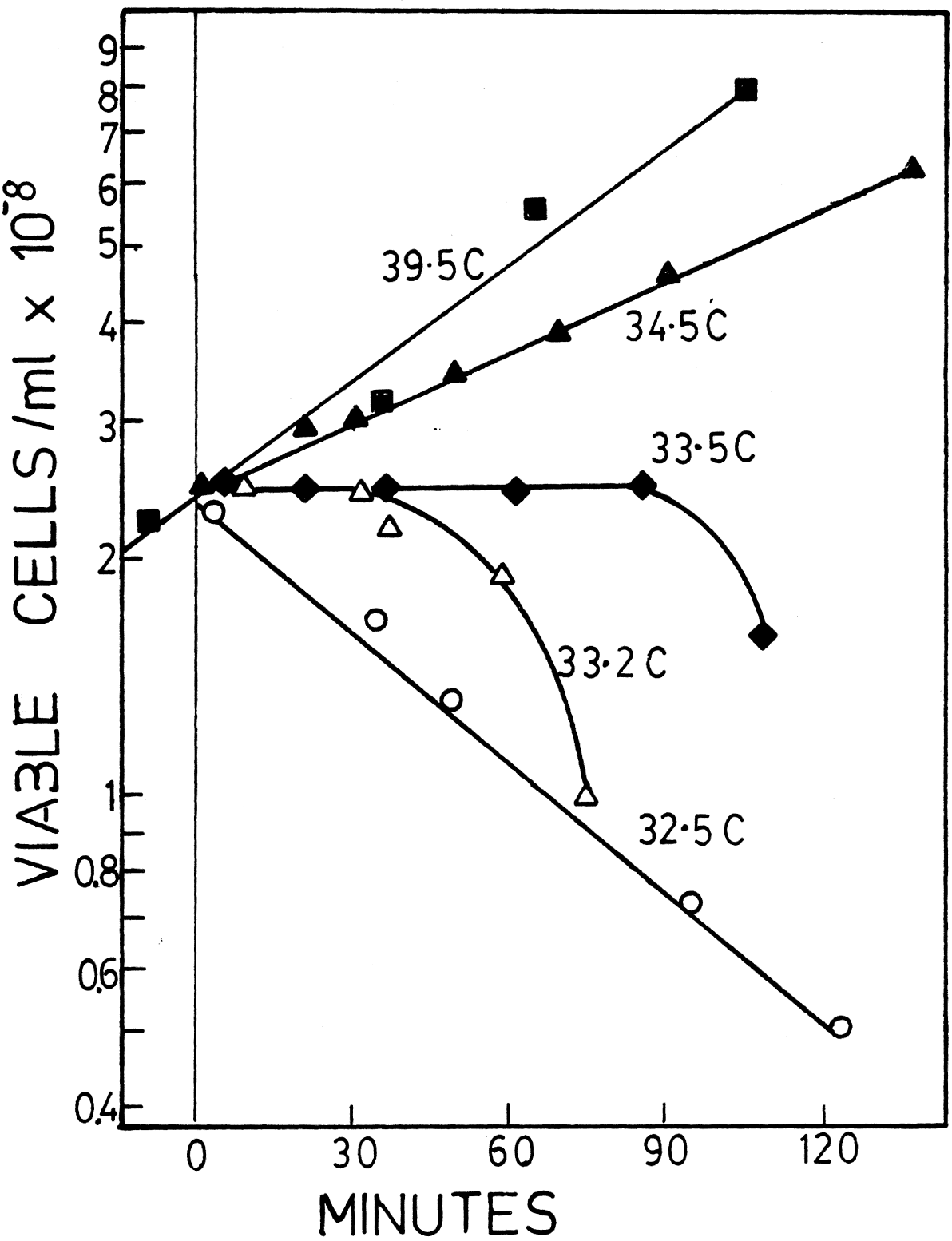

VIABLE CELLS/ml x 10⁸
5
4
3
2
1
0.9
0.8
0.7
0.6
0.5
0.4
0
30
60
90
120
MINUTES

B. Temperature Shifts Above 30C: Membrane Phase Transition in Viable Cells.

A solution to the obstacle presented in the previous section existed in the possibility that elaidate-grown cells of strain K1059 might be capable of surviving temperature shifts at temperatures between 30 C and some higher temperature that was still within the range of temperatures causing membrane phase transition. Based on the observations of Overath et al. (281) and of Plate (294), elaidate-grown Ufa^- auxotrophs should be capable of surviving temperatures above 30 C while still exhibiting alterations in membrane-related functions at temperatures as high as 37 C.

Using the same basic procedure for initiating temperature shifts, as described above, viability was measured at a number of temperatures between 30 C and 37 C (Figure 22). The results indicate that cell viability could be maintained in the presence of MSCBE containing 0.5% glycerol at certain temperatures where the initiation of membrane phase transition results in cessation of increase in optical density and cell number (Figure 22). Temperatures of 33.2-34.0 C allowed survival for a minimum of 30 minutes without loss of viable cell number, whereas temperatures below 33.2 C resulted in rapid cell death. When the temperature shift was to temperatures of 34 C and above in the presence of glycerol there was only a reduction in the rate of accumulation of cell number and cell mass.

Figure 22. The Effect of Temperature Shifts to Temperatures Above 30 C on Viability of Elaidate-Grown Cells of Strain K1059. Cultures of strain K1059 in MSCBE at 39.5 C were collected upon reaching mid-logrithmic phase of growth and divided into fractions (indicated by the zero minute mark); one fraction remained at 39.5 C (for each experiment) in MSCBE and a second fraction was shifted to one of four temperatures indicated while remaining in MSCBE. Cell number was determined by viable count on MSCBO agar in each case. For each temperature demonstrated, the result depicted is representative of at least two separate experiments.


VIABLE CELLS/ml x 10⁻⁸
9
8
7
6
5
4
3
2
1
0.8
0.6
0.4
39·5C
34·5C
33·5C
33·2C
32·5C
0
30
60
90
120
MINUTES

C. Restrictions on Mating System.

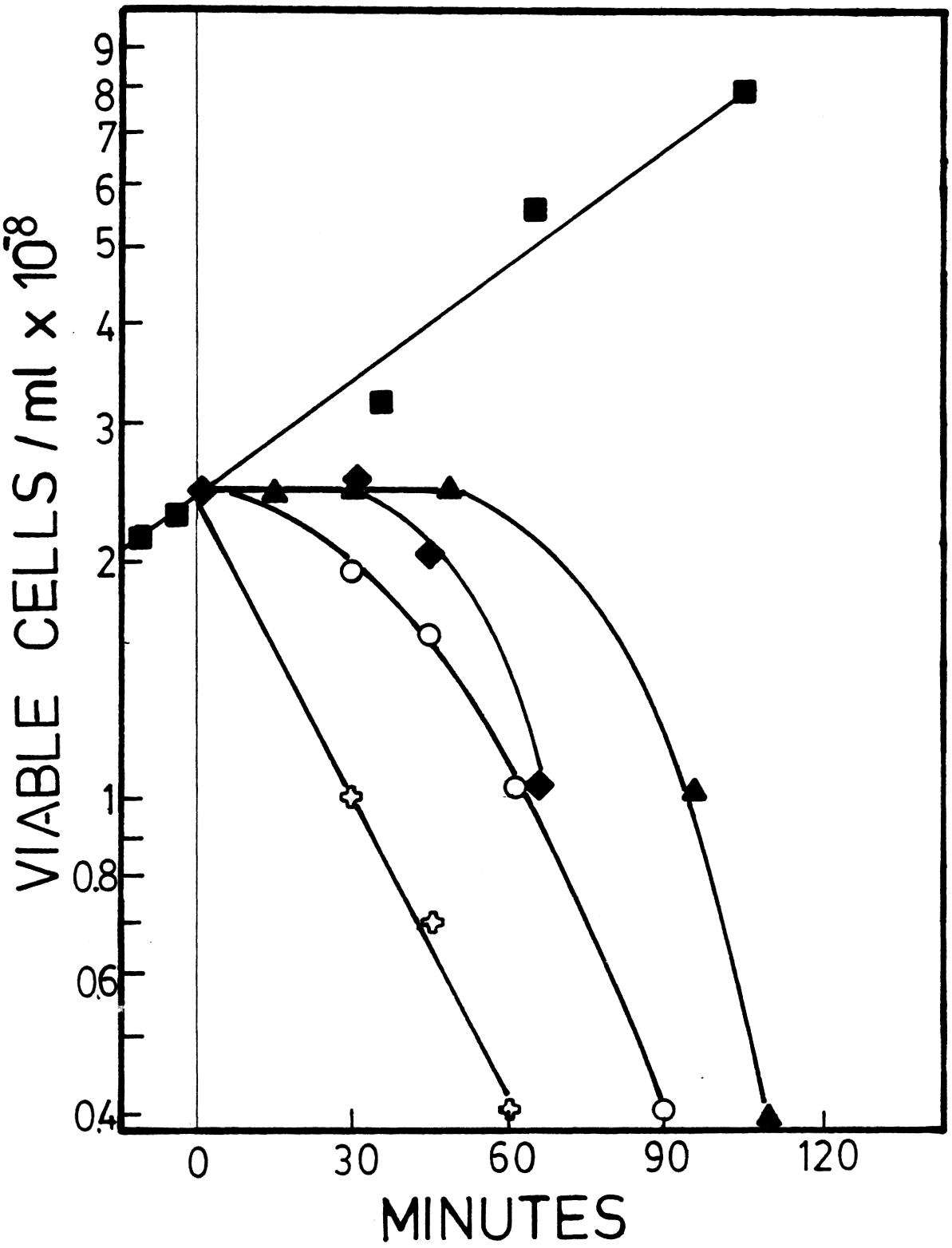
Finding conditions which would allow survival of elaidate-grown cells of strain K1059 simultaneous with the onset of a membrane phase transition suggested that conditions might be met which would permit the use of Ufa$^-$ auxotrophs in the investigation of the effect of the phase transition phenomenon on conjugation. At this point, it was imperative to determine what conditions would suffice to allow survival of elaidate-grown cells of strain K1059, yet still allow for measurable levels of conjugation. It has previously been demonstrated that the availability of an energy source which may be utilized by the donor is required to obtain measurable levels of conjugation (189). Comparison of glycerol versus glucose as the donor energy source indicated that the presence of glucose in the mating mixture resulted in greater transconjugant yields (Table II). Since glucose was thus the obvious choice for use as a carbon source in conjugation, we investigated the ability of elaidate-grown cells of strain K1059 to survive temperature shifts above 30 C in the presence of glucose. The data in Figure 23 indicates that elaidate-grown cells were much more sensitive to the temperature shift in the presence of glucose than in the presence of glycerol. At 34.0 C or below, cells demonstrated logrithmic death rates, whereas cells at temperatures at 34.5 C or above appeared to survive the temperature shift to some extent. Because the use of glucose as the carbon source in the mating mixture would greatly reduce the already narrow range of temperature that could be used in the investigation of membrane phase transition effects

Table II
Determination of χ1886 Conjugation Proficiency Under Variable Conditions of Growth and Mating

(a) Donor Growth Medium	(b) Recipient Growth Medium	(c) Recipient suspended in buffer	(d) Relative transconjugant yield
MSC glucose	MSC glucose		
		-	100%
		+	100%
	MSC glycerol		
		-	100%
		+	100%
	MSCBO		
		-	0.09%
		+	136%
MSC glycerol	MSC glycerol		
		-	41%
		+	39%
	MSCBO		
		-	0.07%
		+	43%
MSCBO	MSCBO		
		-	0.005%
		+	0.012%

χ1886 was grown at 37 C in one of three media as indicated (a) and then mated with an F^- recipient strain, χ59, grown at 37 C in one of the listed media for recipient (b). For the matings (37 C) χ59 cells were either left in medium of growth or were first collected on membrane filters and suspended in MS (37 C) prior to mixing with donor cells (c). Relative transconjugant yields are measured against the value obtained from the mating between donor grown in MSC-glucose and the recipient grown in MSC-glucose where the recipient remained in medium of growth for the mating (d).

Figure 23. The Effect of Glucose on Cell Viability at Temperatures Above 30 C of Elaidate-Grown Cells of Strain K1059. Cultures of strain K1059 in MSCBE at 39.5 C were collected upon reaching mid-logrithmic phase of growth (indicated by the zero minute mark) and divided into fractions; one fraction was maintained in MSCBE at 39.5 C (■) while the remaining fractions were filtered and suspended in MSCBE containing 0.5% glucose (as opposed to 0.5% glycerol) followed by temperature shifts to either 32 C (✣), 34 C (○), 34.5 C (◆), or 35 C (▲). Cell number was estimated by viable count on MSCBO agar. Result depicted is representative of two separate experiments.


VIABLE CELLS / ml x 10⁸
9
8
7
6
5
4
3
2
1
0.8
0.6
0.4
0
30
60
90
120
MINUTES

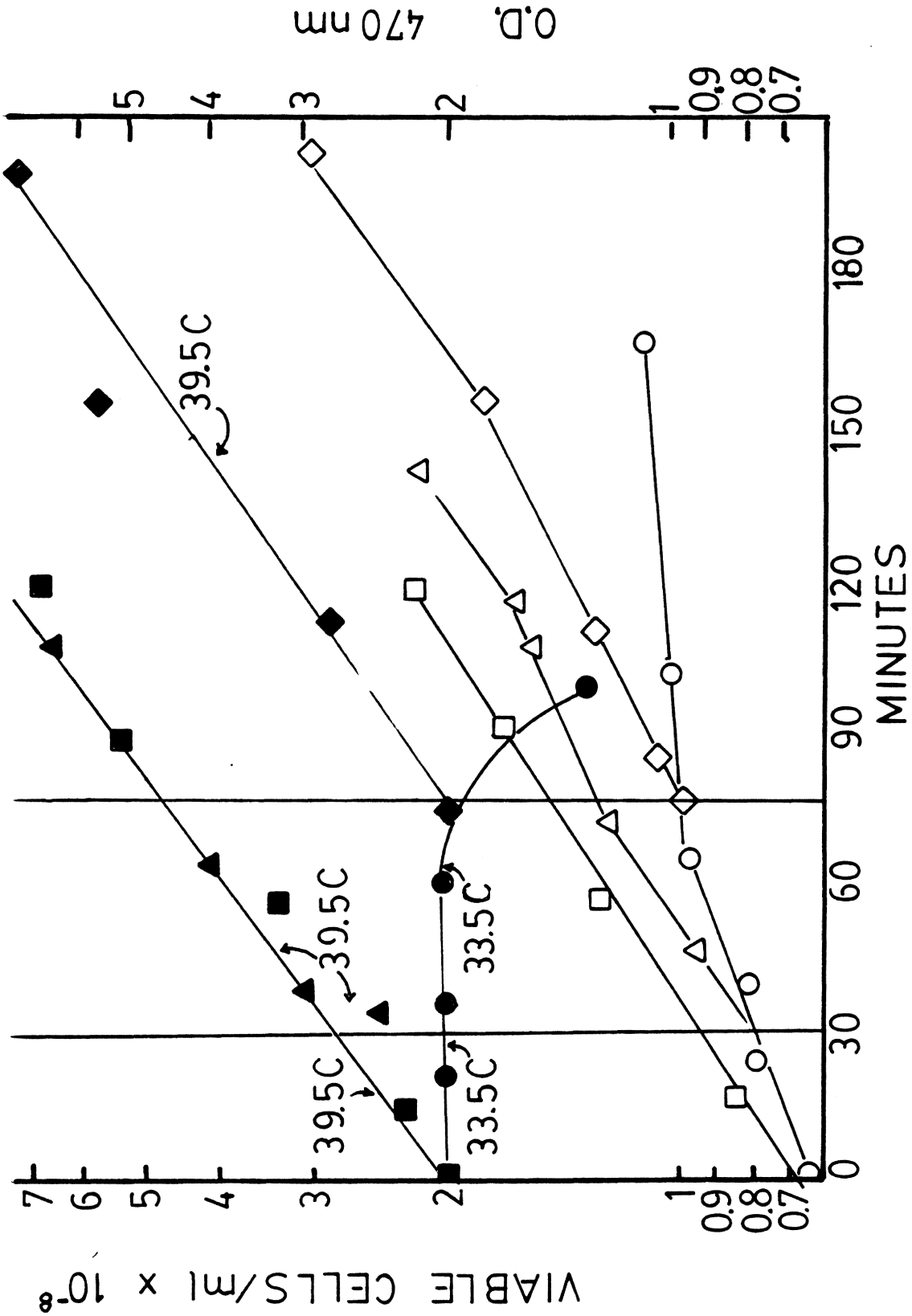
on conjugation, it was decided that glycerol could serve as a suitable carbon source for the purpose of this investigation.

The results of Table II also indicate that elimination of fatty acids from the mating mixture is required to maximize transconjugant yield. Conjugation proficiency in the presence of the non-ionic detergent, Brij 35, was measured to determine if its presence might affect protein (pili: receptor interaction) function as is often the case with non-ionic detergents (1, 354). The data in Table II clearly indicate that Brij 35 does cause greatly reduced levels of conjugation. The absence of Brij 35 from the mating mixture negates the use of fatty acids in the mating mixture since elaidate and oleate demonstrate very different degrees of solubility in aqueous solutions at low temperatures. This, however, should not affect the ability of strain K1059 and its variants to survive during a 30 minute mating period since it has been demonstrated, here in this report, and by others (158) that Ufa$^-$ auxotrophs may survive over an hour in the absence of unsaturated fatty acids.

D. Development of a Mating System.

The next step in the development of a mating system required the demonstration that the effect of a temperature-induced membrane phase transition was reversible with a reversal of the temperature shift. Any alteration of *in vivo* cell metabolism is likely to be reflected in the cells ability to grow and divide; thus, measurement of cell mass and viable cell number served to determine the effects of a double temperature shift (Figure 24) on cell function. Cells of strain

Figure 24. Determination of the Reversibility of the Effects of Temperature Shifts on Elaidate-Grown Cells of Strain K1059. A culture of strain K1059 in MSCBE at 39.5 C was collected and divided into two fractions upon reaching the mid-logrithmic phase of growth (indicated by the zero minute mark). Still in MSCBE, one fraction remained at 39.5 C (■ , □) and the second fraction was shifted to 33.5 C (● , ○). Fractions of the suspension at 33.5 C were removed and shifted back to 39.5 C at 30 minutes (▲, △) and at 75 minutes (◆, ◇) following the initial shift to 33.5 C. Cell number was determined by viable count on MSCBO agar (closed symbols) and cell mass was estimated by optical density determination at 470 nm (open symbols) following 1:10 dilution in MS. Results depicted are representative of three separate experiments.


VIABLE CELLS/ml x 10^{-8}
MINUTES
O.D. 470 nm
39.5C
39.5C
39.5C
33.5C
33.5C

K1059 grown in MSCBE at 39.5 C and then subjected to a temperature shift to 33.5 C without resuspension immediately ceased to divide, simultaneous with reduced rates of mass increase (cells filament; unpublished observation). At 33.5 C, cells were maintained in MSCBE without loss of viability for periods in excess of 60 minutes (maximum of 75 minutes). Cells shifted back to 39.5 C after 30 minutes at 33.5 C increased in cell number and mass rapidly. Within a matter of minutes they reached cell numbers identical to control cells which were not subjected to the temperature shift (Figure 24). Cells shifted back to 39.5 C after 75 minutes did not exhibit the same rapid increase in cell number, but did resume cell division immediately upon exposure to the growth permissive temperature and did so at a rate comparable to that of cells growing continuously at 39.5 C (Figure 24).

Because Brij 35 inhibited conjugation (Table II), successful mating depends on the ability of strain K1059 cells both to survive mating in the absence of unsaturated fatty acids and Brij 35 and to resume growth when an unsaturated fatty acid is again made available to the cells. The capacity of strain K1059 cells to meet this requirement was demonstrated as follows. MSCBE-grown cells of strain K1059 were filtered and suspended as if in preparation for a mating. One minute following a temperature shift to 33.5 C, an equal volume of donor growth medium (MSC-1% glycerol) prewarmed to 33.5 C was added to the strain K1059 cell suspension to mimic the addition of donor cells. Cells were then incubated for 30 minutes at 33.5 C and then part of that sham mating suspension was filtered and suspended MSCBE prewarmed

to 39.5 C. The results (Figure 25) demonstrated that strain K1059 cells can survive a temperature shift to 33.5 C (under mating conditions in the absence of donor cells) and resume growth following an elevation in growth temperature to 39.5 C in complete medium.

As a final precaution, strain K1059 cells grown in the presence of elaidate at 39.5 C were challenged to survive conditions which closely simulated the plating step required for selection of transconjugants (Figure 26). Following a sham mating identical to to the one described above, cells were filtered and suspended in MSCO-1% glucose (instead of MSCBO) to simulate the plating step. No death occurred in these cells and growth resumed at approximately 15 minutes following the shift to 39.5 C (Figure 26). Furthermore, if after the sham mating period cells were immediately brought to 22.5 C for eight minutes in the presence of MSCO-1% glucose (again simulating potential plating procedure where plates were of temperature below 39.5 C) before being brought slowly to 39.5 C no cell death occurred and increase in cell number is observed (Figure 26).

E. Lambda Lysogeny of Strain K1059.

Assured of having obtained a mating system which would allow use of strain K1059 when subjected to membrane phase transition, it was necessary to construct certain variants of strain K1059 to permit investigation into which stage of conjugation might be most affected by altered membrane lipid state. One technique often used to detect the extent of DNA transfer is zygotic induction (183, 250). In the absence of lambda bacteriophage repressor, the lambda genes

Figure 25. Determination of Reversibility of the Effect of a Temperature Shift to 33.5 C from 39.5 C Under Mating Conditions for Elaidate-Grown Cells of Strain K1059. A culture of strain K1059 in MSCBE at 39.5 C was collected upon reaching the mid-logrithmic phase of growth (as indicated by the zero minute mark) and divided into two fractions. One fraction remained at 39.5 C in MSCBE (■ , □) and the remaining fraction was filtered and suspended at 39.5 C in MS (2-fold concentration) followed by a temperature shift to 33.5 C and the addition of an equal volume of MSC-1% glycerol (33.5 C) to simulate the addition of donor (● , ○). After 30 minutes, the portion of cells at 33.5 C was divided into two fractions, one fraction remaining at 33.5 C in mating medium (circles continued) and the second fraction filtered and suspended in MSCBO at 39.5 C (▲ , △). Cell number was determined by viable count on MSCBO agar (filled symbols) and the cell mass was estimated by optical density measurements at 470 nm (open symbols) following 1:10 dilution in MS. Result depicted is representative of two separate experiments.

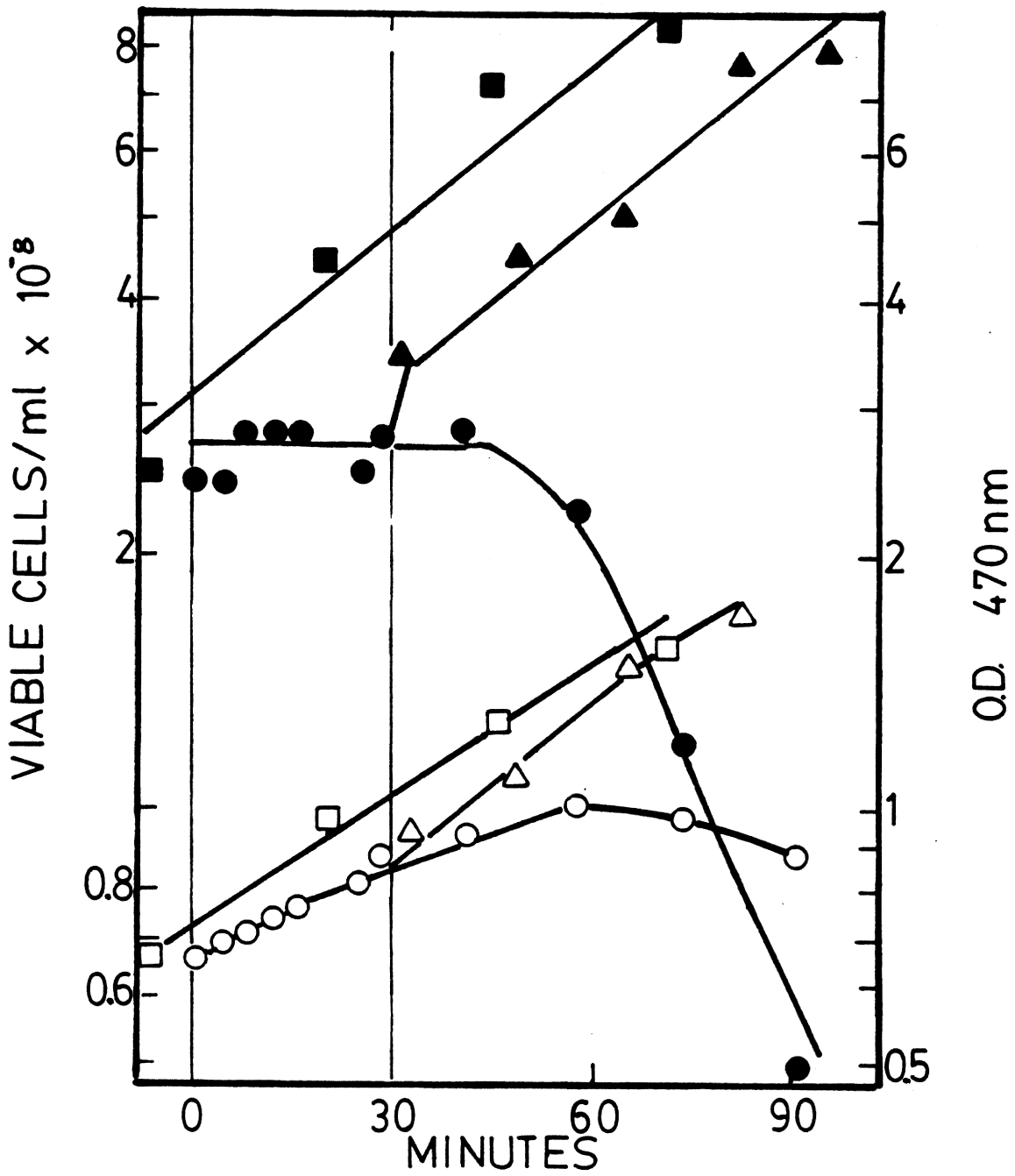
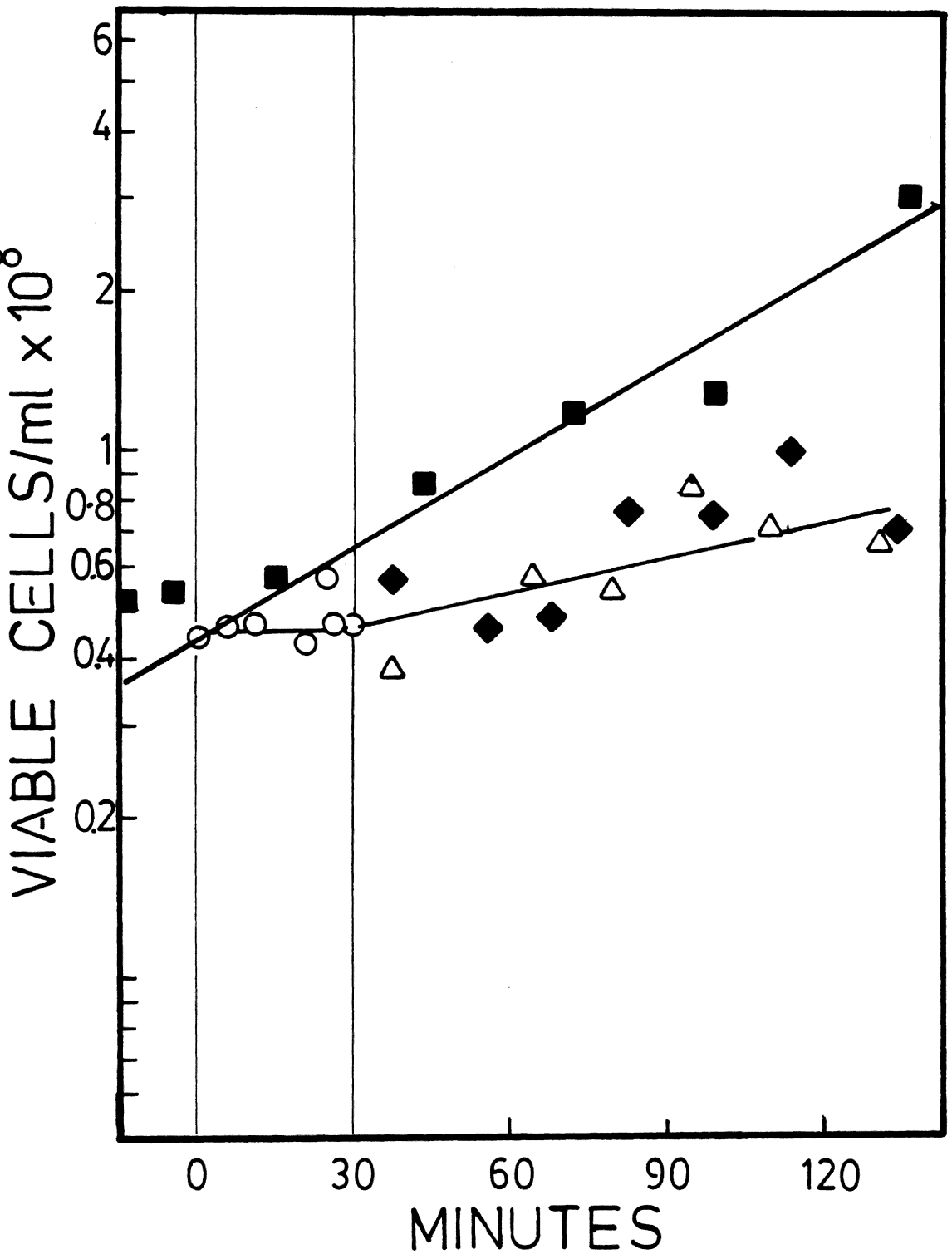

VIABLE CELLS/ml x 10⁻⁸
8
6
4
2
0.8
0.6
0
30
60
90
MINUTES
6
4
2
1
0.5
O.D. 470 nm

Figure 26. The Effect of Mating and Post-Mating Procedures on Viability of Elaidate-Grown Cells of Strain K1059 Subjected to a Temperature Shift and its Reversal. A culture of strain K1059 in MSCBE at 39.5 C was divided into two fractions upon reaching the mid-logrithmic phase of growth (as indicated by the zero minute mark); one fraction remained at 39.5 C in MSCBE (■) and the second fraction was filtered, suspended (2 fold concentration) in MS at 39.5 C, shifted to 33.5 C, and mixed with an equal volume of MSC containing 1% glycerol (33.5 C) to simulate addition of donor cell (○). After 30 minutes under simulated mating conditions that portion of cells at 33.5 C was filtered and suspended in MSBO-1% glucose at either 39.5 C (△) or at 22.5 C prior to a gradual shift to 39.5 C over a 20 minute period (◆). Cell number was determined on MSCBO agar for all fractions. Results are representative of two separate experiments.


VIABLE CELLS/ml x 10^-8
6
4
2
1
0·8
0·6
0·4
0·2
0
30
60
90
120
MINUTES

responsible for cell lysis (transferred from donor) are derepressed in the non-lysogenic recipient following transfer by conjugation (183, 250) and the resulting increase in free lambda phage can be used as a measure of transferred DNA. Use of this technique requires that the recipient be non-lysogenic and resistant to infection by lambda bacteriophage. Upon initiation of procedures to isolate a lambda phage-resistant variant of strain K1059, it was found that strain K1059 was already immune to lambda bacteriophage.

Immunity to infection by bacteriophage lambda generally indicates either cells lack a functional receptor for lambda phage or are lysogenic for lambda (15). The latter proved to be the case. Cells of strain K1059 were sensitive to the virulent lambda mutant, λvir and capable of giving rise to lambda bacteriophage plaques on LB-oleate agar medium after UV irriadiation ($1.2 J/m^2/sec$). Phage production was detected by pouring 1×10^7 cells of Escherichia coli K-12 strain χ59 suspended in Lambda Top Agar on to the surface of the plate containing approximately 100 UV-irriadiated colonies of strain K1059 and then looking for cleared zones around the colonies of strain K1059 in the developing lawn of χ59. Sensitivity to virulent lambda phage and the generation of lambda bacteriophage upon exposure to UV-light fulfill the criteria defining lambda lysogeny (15).

Realization of the lambda lysogeny of strain K1059 allowed for the possibility that the temperature shift-induced loss in cell viability of elaidate-grown cells of strain K1059 might be associated with induction of lambda bacteriophage (220). If the temperature shifts do

result in lambda phage induction the number of free phage in the medium should increase relative to the number of viable cells (212, 378). To determine if this occurred, the viable cell number determined by colony forming units was compared with the number of released phage through the period of a temperature shift to 33.5 C and back to 39.5 C under simulatated mating conditions for both MSCBO- and MSCBE-grown cells (Figure 27 and 28). The results indicated that the ratio of free phage to cell number is constant through both temperature shifts in both MSCBE- and MSCBO-grown cells of strain K1059. Consequently then, the fact that strain K1059 is a lambda lysogen would have no effect on matings with λ^{vir} -resistant donor cells.

F. Effect of Membrane Phase Transition on Transconjugant Formation.

Because the formation of transconjugants (recipient cells which express the genes on the DNA transferred from the donor cell) requires that all the functions of conjugation are operational, a measure of the formation of transconjugants in cells subjected to membrane phase transition should indicate if any of the steps of conjugation are affected by the altered membrane phenomenon. I chose to measure transfer and establishment of the plasmid R-100-1 for a number of reasons. Should membrane phase transition cause uncoupling of energy metabolism, this plasmid can be inherited from the donor by conjugation in the absence of energy expenditure by the recipient cell (65, 66, 89). Additionally, transfer and establishment of plasmid DNA does not require recombination events in the recipient and thus the complex cellular metabolism involved in this process (61, 63) is avoided.

Figure 27. The Effect of a Temperature Shift and its Reversal on the Release of Free Lambda Bacteriophage From Oleate-Grown Cells of Strain K1059. A culture of strain K1059 in MSCBO at 39.5 C was collected upon reaching the mid-logrithmic phase of growth (indicated by A at 0 minute mark) and divided into three fractions; one fraction remained at 39.5 C in MSCBO (▲ , △) while the other fractions were suspended in MSC-0.5% glycerol (39.5 C) followed by a temperature shift to either 36.5 C (◆ , ◇) or 33.5 C (● , ○) for 30 minutes. At 30 minutes, a temperature shift reversal was effected (back to 39.5 C) simultaneous with suspension in MSCBO as indicated by the mark at B). Viable cell number was determined by colony forming ability on MSCBO agar (filled symbols) and free lambda bacteriophage were monitored by the formation of plaques on a lawn of the lambda sensitive strain, X59 (open symbols). Result depicted is representative of two separate experiments.

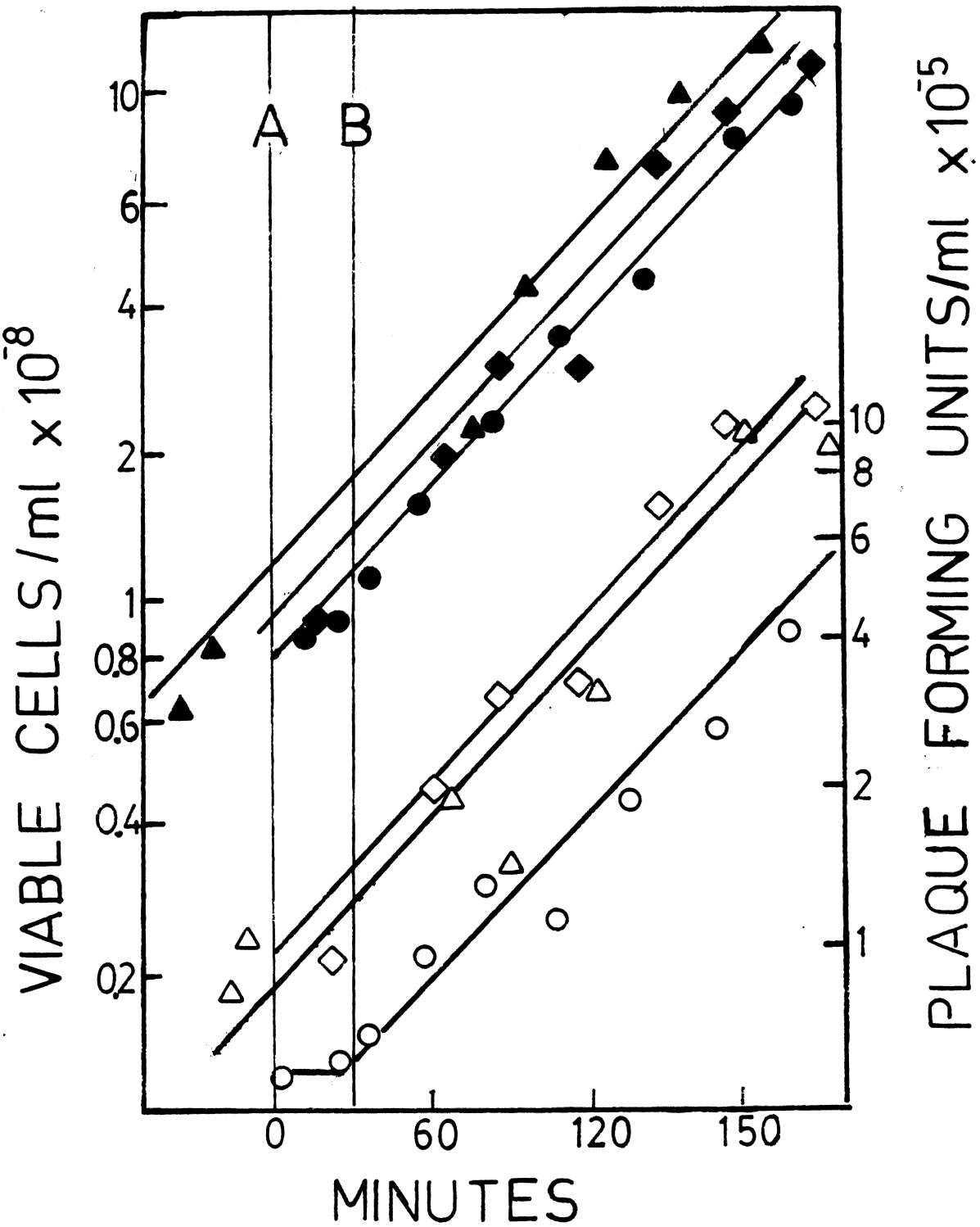
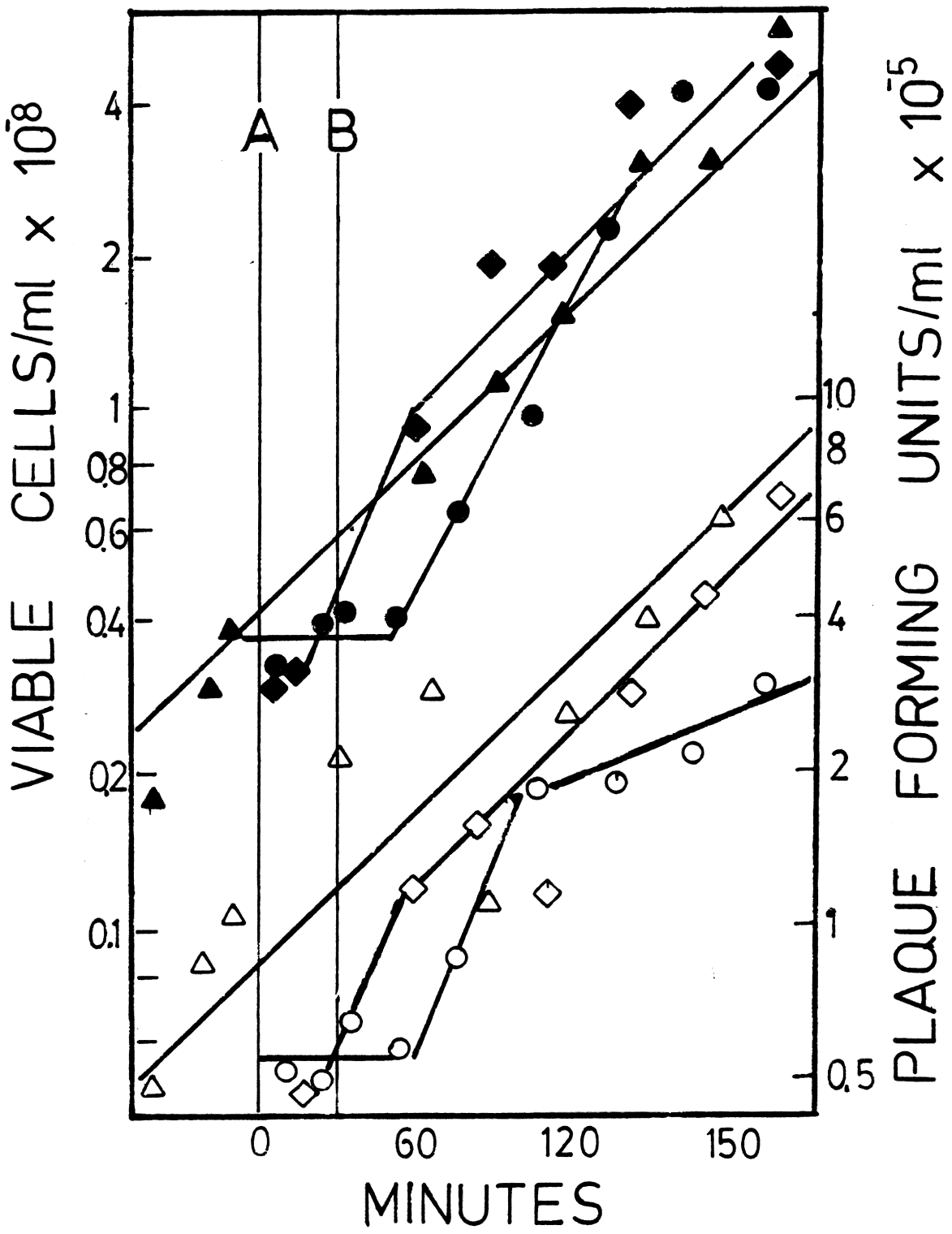

A
B
10
6
4
2
1
0.8
0.6
0.4
0.2
VIABLE CELLS/ml x10⁻⁸
10
8
6
4
2
1
PLAQUE FORMING UNITS/ml x10⁻⁵
0
60
120
150
MINUTES

Figure 28. The Effect of a Temperature Shift on the Release of Free Lambda Bacteriophage From Elaidate-Grown Cells of Strain K1059. A culture of strain K1059 in MSCBE at 39.5 C was collected upon reaching the mid-logrithmic phase of growth (indicated by the A at 0 minute mark) and divided into two fractions; one fraction remained at 39.5 C in MSCBE (▲, △) while the other fractions were suspended in MSC-0.5% glycerol (39.5 C) followed by a temperature shift to either 36.5 C (◆, ◇) or 33.5 C (●, ○) for 30 minutes. At 30 minutes, a temperature shift reversal was effected (back to 39.5 C) simultaneous with suspension in MSCBO (indicated by mark B). Viable cells count was determined by colony forming ability on MSCBO agar (closed figures) and free lambda bacteriophage were estimated by the formation of plaques on a lawn of lambda sensitive strain χ59 (open figures). Result depicted is representative of two separate experiments.


VIABLE CELLS/ml x 10^8
PLAQUE FORMING UNITS/ml x 10^{-5}
A
B
4
2
1
0.8
0.6
0.4
0.2
0.1
10
8
6
4
2
1
0.5
0
60
120
150
MINUTES

Finally, transconjugants possessing R100-1 can be easily detected by the incorporation of antibiotics in media and expression of resistance is unaffected by the presence of Brij 35 and oleic acid (data not shown).

In Table III and Figures 29 and 30 are shown the results from the experiments designed to examine the effect of membrane lipid phase transition in recipient cells on the formation of transconjugants. Each point in Figures 29 and 30 represents the average of 5 or more separate matings. Matings were 30 minutes long followed by selection of transconjugants on MS agar plates supplemented with 0.02% oleate, 0.2% Brij 35, 1% glucose, and 50 ug streptomycin/ml. It is apparent that for cells of strain K1059, the number of transconjugants formed by elaidate-grown cells relative to oleate-grown cells is significantly reduced at temperatures below 35 C.

In the parent of strain K1059, Escherchia coli K-12 strain Ymel, and in an unrelated K-12 variant, ψ59, the presence of elaidate or oleate during growth has no effect on the relative levels of transconjugants formed at any temperature between 39.5 C and 33.5 C. It thus appears that some step in conjugation is affected by the occurrence of a membrane lipid phase transition, as is indicated by the reduced levels of transconjugants arising from elaidate-grown cells of strain K1059 at temperatures below 35 C relative to transconjugant yield in arising from oleate-grown cells of strain K1059.

Table III
Effect of Recipient Cell Membrane Phase on
Transconjugant Yield

Mating Temperature	(a) Transconjugant Yield in Recipient Cells; elaidate-supplemented relative to oleate-supplemented		
	Strains	(b)	
	Ymel	K1059	59
39.5 C	123%	140%	97%
37.0 C	80%	99%	120%
35.0 C	123%	107%	92%
34.0 C	95%	31%	N.D.
33.5 C	110%	9%	91%

Matings were conducted as described in Materials and Methods. All matings were of 30 minute duration and conducted at the temperature indicated following suspension of recipient in MS (39.5 C) and the shift to mating temperature. The donor strain, OT135, was grown at temperature of eventual mating in MSC-1% glycerol. Recipient strains were grown in either MSCBO or MSCBE at 39.5 C regardless of the need for unsaturated fatty acids supplementation. Relative transconjugant yield is the percent ratio of transconjugant yield in elaidate-grown cells relative to the transconjugant yield with oleate-grown cells (a). Each ratio is the average of a minimum of 5 separate matings. Reversion of the Fab^- and Fad^- phenotypes monitored for each mating with K1059 (b).

Figure 29. Arrhenius Plot Representation of Transconjugant Yield Versus Mating Temperature for Matings Between Strain OT135 and Strain K1059 Grown with Oleate or Elaidate. Details given in Materials and Methods and in Figure 3. The diagram is meant to function only as an extension of the information presented in Table III. The rate of reaction (plasmid transfer), estimated by the number of transconjugants yielded in 30 minute matings, is plotted against the reciprocal of the absolute temperature of the mating. The figure allows visualization of the extensive decrease in recipient ability over a small temperature increment for elaidate-grown cells (■) relative to transconjugant yield in oleate-grown cells (○) of strain K1059. OT135 served as donor. Results depicted are compiled from a minimum of 10 matings at each temperature for which there is a figure.

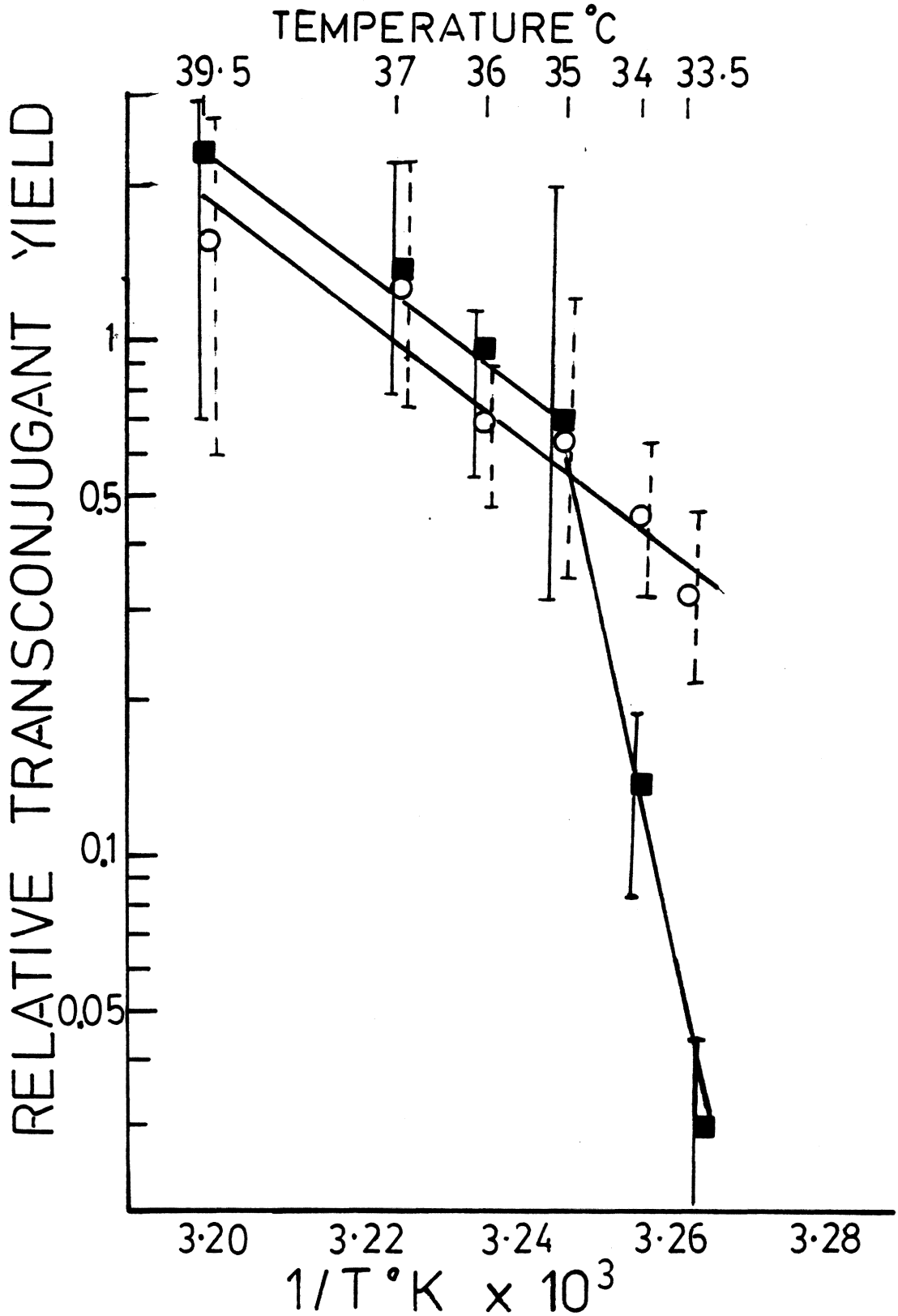
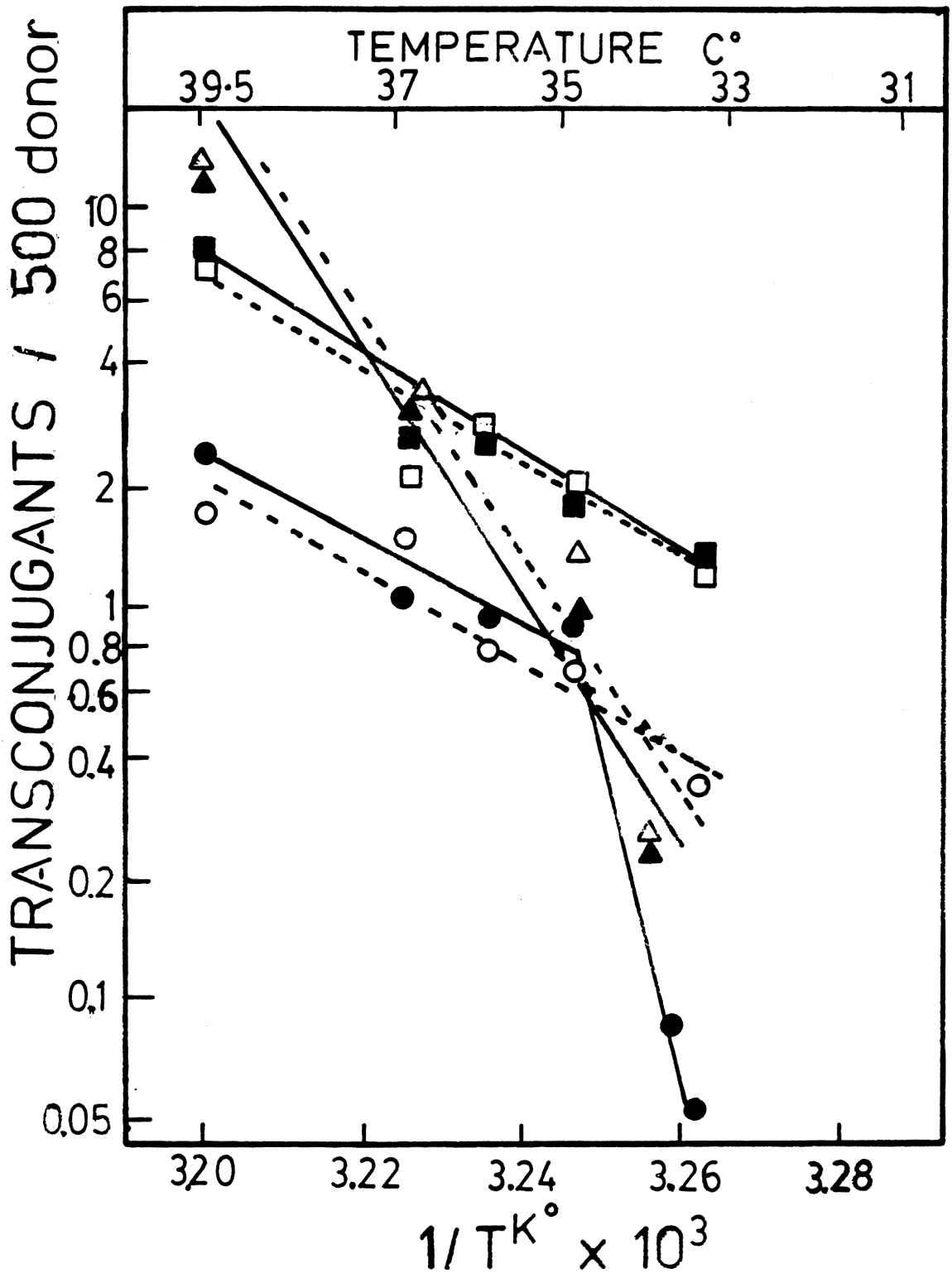

TEMPERATURE °C
39·5
37
36
35
34
33·5
RELATIVE TRANSCONJUGANT YIELD
1
0.5
0.1
0.05
3·20
3·22
3·24
3·26
3·28
1/T °K x 10³

Figure 30. Arrhenius Plot Representation of Transconjugant Yield in Matings with Donor Strain OT135 and Recipient Strains Ymel, K1059, and 59 Following Growth in MSCBO or MSCBE. Details of mating procedure in Figure 3 and Materials and Methods. Diagram is meant only as a supplement to Table 3. Rate of reaction (transfer of plasmid) estimated by the number of transconjugants generated in a 30 minute mating, is plotted against the inverse of absolute temperature of mating with donor strain OT135. Data represents matings with Ymel following growth in in MSCBO (△) or MSCBE (▲), ψ59 following growth in MSCBO (□), or MSCBE (■), and K1059 following growth in MSCBO (○) or MSCBE (●). Each data point represents an average of at least 5 separate mating experiments.


TEMPERATURE C°
39·5
37
35
33
31
TRANSCONJUGANTS / 500 donor
10
8
6
4
2
1
0.8
0.6
0.4
0.2
0.1
0.05
3.20
3.22
3.24
3.26
3.28
1/ T^K° x 10^3

G. Mating Termination With T6 Bacteriophage.

Generally, the selection of transconjugants immediately following DNA transfer frequently results in the detection of fewer transconjugants than could actually be formed. Apparently the recipient cell must fulfill a number of yet undefined requirements following DNA transfer to allow expression of the newly received genes (120). It has been noted by some investigators that these requirements may often be served by exposing the putative transconjugants to complex medium either prior to, or simultaneous with the challenge to these cells of expression of transferred determinants (140, 183, 308)

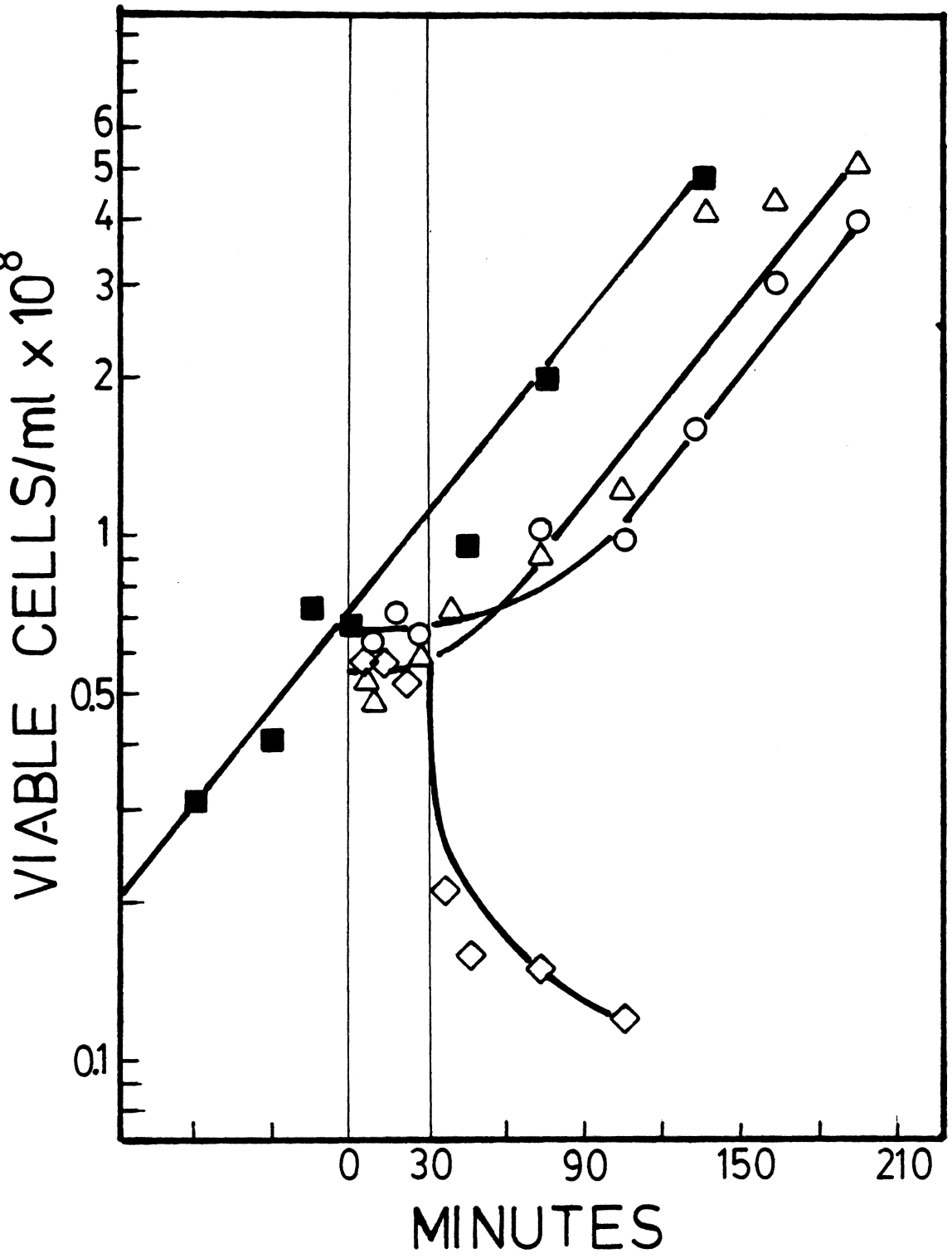
It is possible that the resistance determinants associated with R factor R100-1 transferred to elaidate-grown cells of strain K1059 when subject to membrane lipid phase transition are not expressed as readily as they are when transferred to oleate-grown cells of strain K1059 at the same temperatures. Because elaidate-grown cells subjected to a 30 minute period at 33.5 C readily recover normal growth characteristics within minutes of a shift back to 39.5 C (see Figure 24) it may be assumed that a short period of post-mating incubation should suffice in allowing sufficient recovery for functions required for expression of newly received plasmid genes.

To test this possibility, a mating system was devised which allowed selective removal of the donor cell from the mating mixture and also permitted a post-mating incubation period for transconjugants prior to selection for genes transferred during the mating. To fulfill requirements of this mating system a R100-1 donor strain sensitive to

T6 bacteriophage and resistant to lambda phage (strain OT155) and a T6 resistant variant of strain K1059 (strain OT196) were used in matings. The T6 sensitivity of strain OT155 allowed selective and rapid removal of this donor from the mating mixture. In order to allow maximum adsorption of the T6 bacteriophage to the donor, it was necessary that the incubation with T6 occur in the absence of Brij 35 and unsaturated fatty acids. It was initially assumed that this would not prove an obstacle for it had been demonstrated that unsaturated fatty acid auxotrophs could survive for hours in the absence of unsaturated fatty acids (158). These cells apparently synthesizing phospholipids which contain two saturated fatty acids as opposed to the normal phospholipid complement of one saturated and one unsaturated fatty acid. However, the results of the experiment depicted in Figure 31 indicated that elaidate-grown cells of unsaturated fatty acid auxotrophs, which were subjected to a 30 minute temperature shift to 33.5 C in the absence of unsaturated fatty acids, did not readily survive a reversal of the temperature shift induced membrane phase transition when suspended in medium lacking unsaturated fatty acids and containing glucose.

The onset of cell death of elaidate-grown cells of strain OT196 following a reversal of the temperature shift may be reflection of the requirement for some minimal level of membrane fluidity for maintenance of cellular integrity in cells starved for unsaturated fatty acids (72). In the case of our experiment described in Figure 31, the Ufa^- cells grown with elaidate and starved for unsaturated fatty acids for 30

Figure 31. The Effect of a Double Temperature Shift in the Absence of Unsaturated Fatty Acids on Viability of Elaidate-Grown Cells of Strain OT196: Preparation for T6 Bacteriophage Terminated Matings. A culture of strain OT196 in MSCBE at 39.5 C was divided into four fractions upon reaching the mid-logrithmic phase of growth (indicated by the zero minute mark); one fraction remained at 39.5 C in MSCBE (■) and the remaining fractions were filtered, suspended in MS (2 fold concentration), and shifted to 33.5 C in the presence of an equal volume of MSC-1% glycerol (33.5 C) for 30 minutes. Following the thirty minute temperature shift, two samples at 33.5 C were further supplemented with either 0.02% oleate and 0.2% Brij35 (final concentration) (△) or 0.02% elaidate and 0.2% Brij35 (final concentration) (○). The remaining fraction (◇) received no additional supplement but was added directly to 10-fold volume excess of Luria Broth containing the T6 bacteriophage. Following the change in medium components, all three fractions were shifted back to 39.5 C and viable count was continued on MSCBO agar.


VIABLE CELLS/ml x 10⁻⁸
6
5
4
3
2
1
0.5
0.1
0
30
90
150
210
MINUTES

minutes could have membranes in which the fluidity has fallen below the minimum fluidity requirement very shortly after the temperature shift reversal into rich medium containing glucose.

Compensation of the limitations defined by the results of the experiment outlined in Figure 31 did not prove an insurmountable obstacle. Assuming that the onset of cell death of elaidate-grown cells of strain UT196 following a temperature shift to 33.5 C for 30 minutes and a reversal of the temperature shift in the absence of unsaturated fatty acids requires active metabolism (even in the presence of ordered-phase membranes) it might be possible to prolong cell viability by suspending cells in medium which will fail to support respiration in the unsaturated fatty acid auxotrophs. Luria broth (LB) contains no readily fermentable carbon source (evidenced by the absence of growth of hemA mutants) and, the addition of non-lethal concentrations of CN^- (10^{-3}M) allow for inhibition of respiration (90). The presence of CN^- at low concentrations does not inhibit expression of newly transferred determinants (90). Furthermore the addition of LB-cyanide mixture to minimal medium plates along with transconjugants allows dilution of CN^- (and its rapid degradation) while complying with the conditions which allow for recovery of a higher proportion of transconjugants as described by Gross (140, 141).

To test Ufa^- cell viability through the mating termination procedures described above, three separate simulated mating experiments were conducted (Table IV). In each case MSCBE- and MSCBO-grown cells of

Table IV
Survival of OT196 Under Conditions Permitting Mating Termination by T6 Bacteriophage Selective Lysis of Donor

(a) Growth Medium	(b) Temperature of simulated mating	(c) Cell number (average) throughout mating (x 10^{-8})	(d) Period of mating termination (minutes)	(e) Cell number at end of mating termination procedure (x 10^{-8})
Experiment I				
MSCBO	39.5 C	4.9	10	4.8
MSCBO	36.5 C	4.9	10	4.8
MSCBO	33.5 C	4.9	10	4.8
Experiment II				
MSCBO	39.5 C	4.9	10	5.4
MSCBO	39.5 C	4.9	10	5.2
MSCBO	33.5 C	4.9	10	4.1
Experiment I				
MSCBE	39.5 C	3.3	10	3.1
MSCBE	36.5 C	3.3	10	3.2
MSCBE	33.5 C	3.3	10	3.3
Experiment II				
MSCBE	39.5 C	3.5	10	4.0
MSCBE	36.5 C	3.5	10	4.3
MSCBE	33.5 C	3.5	10	3.7
Experiment III				
MSCBE	39.5 C	.52	45	.88
MSCBE	33.5 C	.52	45	.81

Three separate experiments demonstrating the ability of elaidate- and oleate-grown cells of strain OT196 to survive mating termination procedures in the absence of unsaturated fatty acids (a). Logrithmic phase cells were subjected to a simulated mating at temperature indicated (b) during which time 5 separated viable counts in triplicate were performed (c). Following a 30 minute mating period, cells were shifted to LB containing 1 x 10^{10} T6 pfu/ml and 5mM KCN for various incubation periods (c) and viable count was then determined in quadrublicate (e).

strain OT196 were prepared for matings as described previously and subjected to a simulated mating at various temperatures. Following the 30 mating period, cells of strain OT196 were suspended in 10-fold excess volume of LB (37 C) containing 1 x 10^9 T6 pfu/ml and 5 mM KCN for periods of either 15 to 45 minutes. The number of cells added to LB medium was determined from an average of 5 viable counts taken during the mating period. Following incubation in LB containing T6 bacteriophage and CN^-, strain OT196 cells were plated for viable count. As the data indicates, no loss of cell viability is noted through the use of this mating termination procedure.

Table V displays data in which the two mating termination procedures were compared relative to their effect on transconjugant formation. The same mating mixture was either disrupted by T6 lysis of donor in the presence of LB and CN^- or by mechanical shear in the presence of MSC-0.5% glycerol. Obviously the number of measurable transconjugants from the low temperature matings does increase significantly with the presence of LB for each of the matings, but the relative ratios of elaidate-to-oleate-grown cells giving rise to transconjugants is maintained regardless of mating termination procedures. At 33.5 C elaidate-grown cells of strain OT196 yield at least 10-fold less transconjugants than oleate-grown cells of strain OT196 regardless of mating termination procedure. For matings at higher temperatures the effect of different mating termination procedures are less pronounced.

Table V

Effect of T6 Termination of Matings on Transconjugant Formation

Growth Medium	Mating Temperature	Addition of T6	Transconjugant yield
MSCBE	39.5 C	-	1.8×10^{-1}
MSCBE	39.5 C	+	2.4×10^{-1}
MSCBO	39.5 C	-	1.0×10^{-1}
MSCBO	39.5 C	+	1.7×10^{-1}
MSCBE	33.5 C	-	4.7×10^{-6}
MSCBE	33.5 C	+	2.4×10^{-4}
MSCBO	39.5 C	-	2.3×10^{-4}
MSCBO	33.5 C	+	2.7×10^{-3}

Matings were performed as described between R-factor donor, $T6^S$ OT155 and elaidate- or oleate-grown cells of strain OT196. From the same mating mixtures samples were removed and subjected to mating termination by either mechanical shear or by selective lysis of the donor with T6 bacteriophage. Transconjugants formed in matings terminated by mechanical shear were plated immediately following mating termination. Transconjugants formed in matings which were terminated by T6 bacteriophage selective lysis of donor were allowed an incubation period in LB (39.5 C) for 15 minutes following mating termination and were plated in a small volume of LB onto the selective medium. Transconjugant yield is expressed as transconjugants per donor.

H. Reversibility of Membrane Phase Transition Effects on Conjugation.

If the membrane phase transition, as opposed to some secondary event, is directly responsible for the altered levels of transconjugant formation then its effect on conjugation should also be reversible as is the case with the effect of a phase transition on growth characterisitics. The data in Table VI indicates that the effect of the membrane phase transition on conjugation is reversible. Strain K1059 cells were grown in either MSCBO or MSCBE and then subjected to a temperature shift to either 33.5 C, 36.5 C or 39.5 C, for periods of either 10 or 30 minutes under conditions which duplicated the mating conditions previously described (except for the addition of donor cells). Upon reversal of the temperature shifts all fractions demonstrated equivalent levels of conjugation proficiency in matings initiated simultaneous with the shift back to 39.5 C.

I. Maintenance of R100-1 in Cells Subjected to Membrane Phase Transition.

Plasmids of certain types, such as R100-1, are believed to require membrane attachment during cell division in order to successfully complete DNA replication and segregation in developing daughter cells (120). Although the cell function of plasmid maintenance is not a step in conjugation, it is a function required for the appearance of transconjugant colonies on selective medium. If the plasmid fails to be maintained during temperature-induced membrane phase transition then it is possible that detection of those cells which have received the plasmid will not occur.

Table VI
Reversibility of the Effects of Membrane Phase Transition on Recipient Ability

Fatty acid supplement	Pre-mating Shift Temperature	Duration of Pre-mating Temperature Shift	Transconjugants Formed in Matings Following Reversal of Temperature Shift Back to 39.5C (a)
Elaidate			
	39.5 C	10 minutes	1.03
		30 minutes	1.29
	36.5 C	10 minutes	0.72
		30 minutes	0.74
	33.5 C	10 minutes	1.28
		30 minutes	1.78
Oleate			
	39.5 C	10 minutes	1.52
		30 minutes	1.05
	36.5 C	10 minutes	1.20
		30 minutes	1.50
	33.5 C	10 minutes	2.38
		30 minutes	1.45

Cultures of strain K1059 at 39.5 C in either MSCBE or MSCBO were collected upon reaching the mid-logrithmic phase of growth and filtered and suspended in MSC-0.5% glycerol at either 39.5 C, 36.5 C, or 33.5 C. At either 10 minutes or 30 minutes post-temperature shift, fractions of the cells were brought back to 39.5 C and mated with strain OT135 in MSC-0.5% glycerol (cells mated in 1:1 ratio 2.0 x 10^8 cells /ml). Transconjugants were selected on MSBCO-1% glucose containing 25ug streptomycin/ml. Transconjugants formed per 500 donors was determined (a). Data is averaged from two separate experiments.

To investigate plasmid stability in cells subjected to membrane phase transition, an R100-1 carrying strain K1059 (strain OT109) was isolated from a mating between cells of strains OT135 and K1059, and then used to determine if any steps of the mating procedure were capable of causing the plasmid to be lost. Cells of variant OT109 were grown in MSCBE at 39.5 C and then shifted to 33.5 C under conditions which duplicated the conditions where Ufa$^-$ cells would first acquire the plasmid by conjugation as in previously described matings. Following a 30 minute period at 33.5 C in MSC-0.5% glycerol, cells were shifted to 39.5 C and then immediately suspended in MSCBO-1% glucose containing 50 ug streptomycin/ml which mimics the plating of recipient cells following conjugation. Prior to and through both temperature shifts cell number was monitored on media which would allow detection of loss in antibiotic resistance. Media used for measurement of colony-forming ability, included MSCBO-agar and MSCBO-agar supplemented with 50 ug streptomycin per ml, 10 ug tetracycline/ml, or both of these at the same respective concentrations (See Figure 32). The data in Figure 33 demonstrates that the plasmid is maintained throughout the duration of the temperature shift and during the growth period following the reversal of the temperature shift. This suggests that once a plasmid is established in a cell, the onset of a membrane phase transition will not cause faulty segregation and loss of the plasmid. However, this result does not imply that alteration of membrane states has no effects on plasmid establishment. It may be possible that single-stranded plasmid DNA transferred to a cell which

Figure 32. R Factor 100-1 and Its Dissociation into RTF and R Plasmids. Although occurring infrequently in Escherichia coli (157), R100-1 is subject to dissociation into two smaller plasmids, one carrying the determinants for antibiotic resistance (r) and the other carrying the determinants for replication and sexuality (RTF). Dissociation most frequently occurs through the two IS1 insertion sequences, thus the tetracycline resistance determinant between the two IS10 insertion sequences remains in association with the RTF plasmid. Dissociation of r from RTF might lead to unequal segregation of the two plasmids upon cell division resulting in a cell population of mixed antibiotic resistance.

The resistance determinants of R100-1 include those for sulfonamide (sul), streptomycin (str), chloramphenicol (cml), fusidic acid (fus), and tetracycline (tet). Determinants for sexuality and replication include the 19 genes comprising the tra operon required for transfer functions, the origin of vegetative DNA replication (oriV), the orgin of conjugal DNA replication (oriT), the structural regulatory genes for expression of transfer functions (finO, finP), the gene for incompatibilty (inc), and the loci with functions related to replication (repA,B,C) (384).

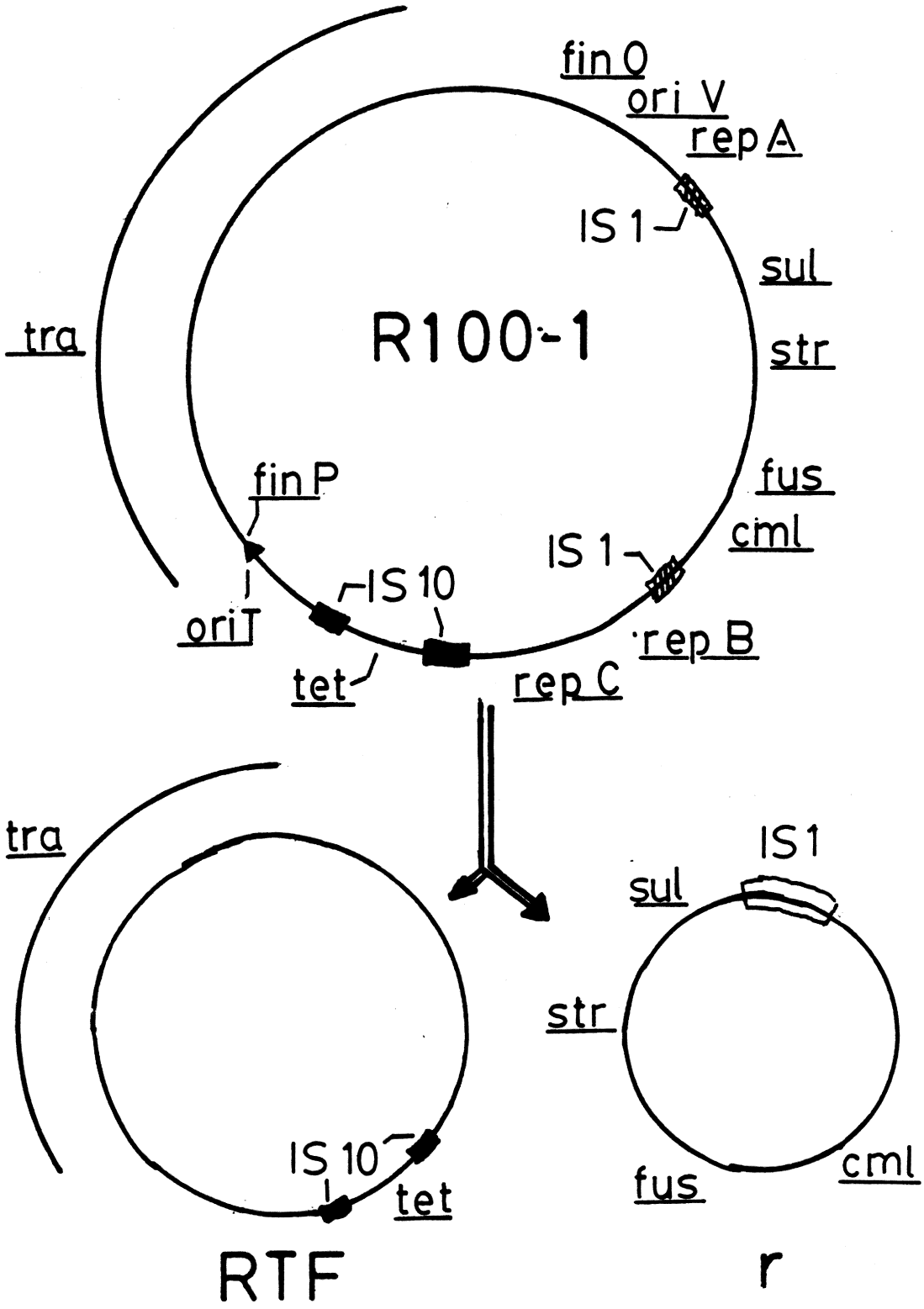
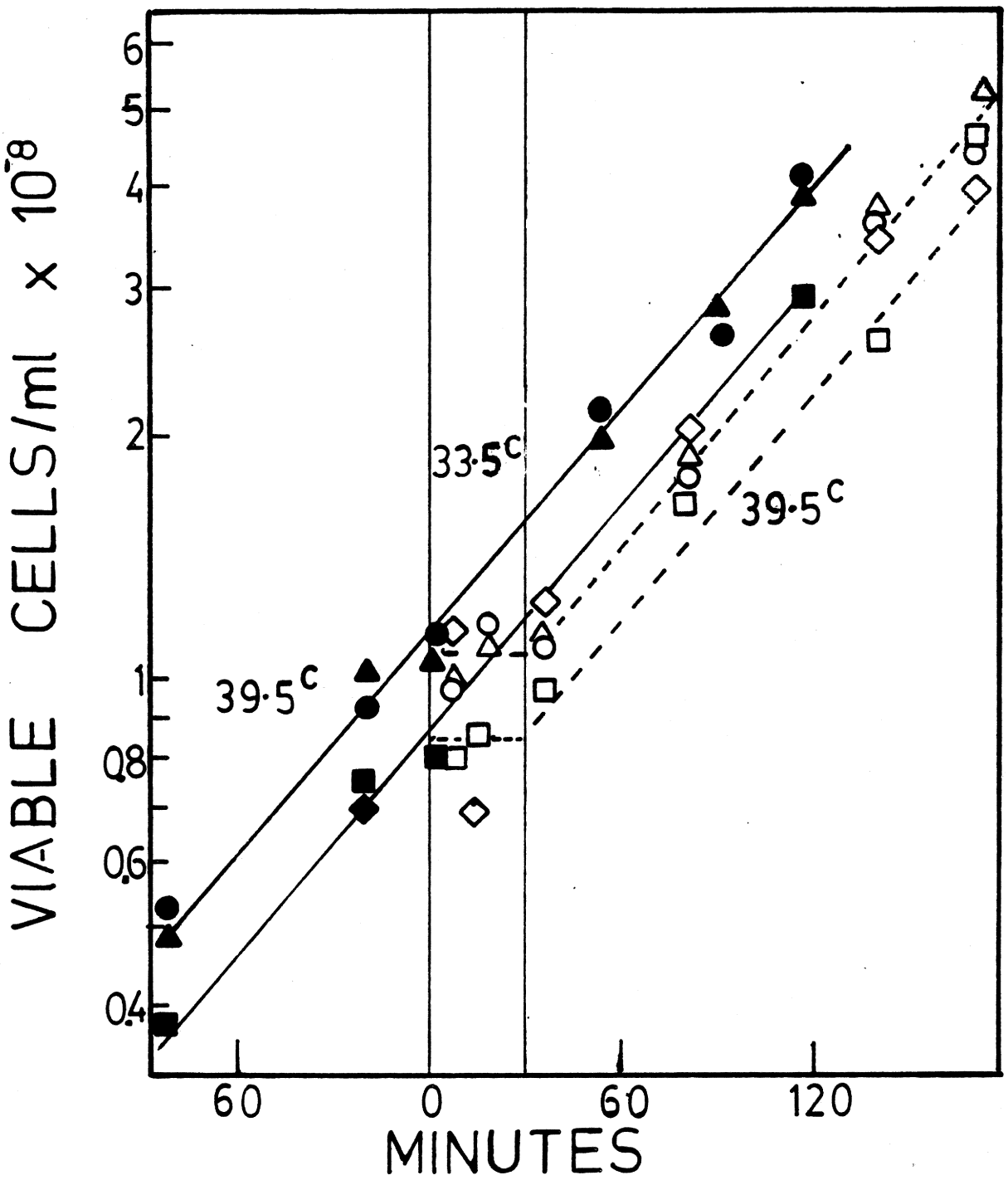

fin O
ori V
rep A
IS 1
sul
str
fus
cml
IS 1
rep B
rep C
tet
IS 10
oriT
finP
tra
R100-1
tra
IS 10
tet
RTF
IS1
sul
str
fus
cml
r

Figure 33. Behavior of R100-1 Plasmid in Cells Subjected to a Membrane Phase Transition. A culture of strain OT198 (K1059 carrying R100-1) at 39.5 C in MSCBE was collected upon reaching mid-logrithmic phase of growth and divided into two fractions (indicated by the zero minute mark); one fraction remained at 39.5 C in MSCBE and the other fraction was subjected to conditions identical to those of the 33.5 C matings previously described with the exception of the addition of donor cells. Cells were filtered and suspended in MS (39.5 C) and then mixed with a 1:1 volume of MSC-1% glycerol (33.5 C) immediately following a temperature shift to 33.5 C. After 30 minutes at 33.5 C cells were brought back to 39.5 C with resuspension in MSCBO-1% glucose Throughout the experiment plasmid stability (maintenance of composite R100-1 structure and normal segregation) was monitored for the cell fraction subjected to the temperature shift (open figures) and the untreated control (closed symbols) by measurement of colony forming ability on MSCBO agar (△, ▲), MSCBO agar supplemented with either 10 ug tetracycline/ml (◇, ◆), 50 ug streptomycin/ml (○, ●) or MSCBO agar supplemented with both 10 ug tetracycline/ml and 50 ug streptomycin/ml. The fraction of the original culture which remained at 39.5 C in MSCBE served as a control through plating on MSCBO agar (□, ■).


VIABLE CELLS/ml x 10⁸
6
5
4
3
2
1
0.8
0.6
0.4
33·5°C
39·5°C
39·5°C
60
0
60
120
MINUTES

is subject to a membrane phase transition cannot successfully associate with its membrane binding site. As a result, the plasmid is not successfully established in the recipient and may possibly be subject to hydrolysis by DNase or even some form of restriction (if double-stranded regions were formed through faulty strand replication or folding within the single stranded DNA) resulting in the formation of DNA fragments or shorter oligonucleotides no longer capable of functioning in transcription.

J. Influence of Membrane Phase Transition on Pair Formation.

Table VII contains the data collected on pair formation as determined by Coulter pair method (119) and the method of of Skurray (110, 335). For both determinations, donor and recipient cells were grown and prepared for mating as in the previously described mating experiments and then mixed for 10 minutes to allow pair formation. The data reflects the measurements of Skurray pairs in four separate experiments, two of which combined simultaneous determination of pairs by Coulter Counter®

Mating pairs as measured by Coulter Counter® indicate that the phase transition in recipient cells has no effect on pair formation. Both oleate- and elaidate-grown cells of strain K1059 demonstrate similar pair formation ability at both 39.5 C and 33.5 C. The small decline in pair formation noted for cells at 33.5 C relative to cells at 39.5 C parallels the decline in mating ability as determined by formation of transconjugants for oleate-grown cells. Quite obviously, the decrease in pair forming ability in elaidate-grown cells between

Table VII
Effect of Membrane Phase Transition on Pair Formation

Donor a	Supplement to K1059	Pair Formation (Mating) Temperature	Pair Formation	
			Coulter®	Skurray
OT135 (+R100-1)	Oleate	39.5 C	52.8%	13.1%
	Elaidate		54.3%	13.7%
	Oleate	33.5 C	34.8%	15.0%
	Elaidate		44.3%	11.8%
OT100 (-R100-1)	Oleate	39.5 C	10.3%	(a) N.D.
	Elaidate		13.0%	N.D.

Cultures of strain K1059 at 39.5 C in MSCBE or MSCBO were collected when in mid-logrithmic phase of growth (2 x 10^8 cells/ml) and prepared for mating as described in Materials and Methods. Recipients were mixed with either strain OT135 or OT100 (the R-factor$^-$, lambda resistant parent of OT155) at either 39.5 C or 33.5 C in 1:1 volume ratios (donor cells at 4 x 10^8 cells/ml) and 15 minutes were allowed to effect pair formation. To serve as controls, fractions of the cultures of the mating participants were incubated in the absence of mating partner under conditions simulating those for the matings. Following the 15 minute incubation, fractions of the donor and recipient controls were mixed in 1:1 volume ratios and then both mated and non-mated mixtures were diluted in MS (membrane filtered) and pair formation determined by either Coulter Counter® technique or by sectored colony formation on LB-oleate agar supplemented with 1% of both lactose, galactose and 50 ug tetrazolium/ml. N.D.; not determined (a). Data collected from two experiments with Coulter Counter® and 10 experiments with Skurray pair formation.

39.5 C and 33.5 C (approximately 20%) does not approach the decrease of transconjugant yield of these cells in the the same range of temperatures (>99%).

The data from pair formation as determined by the Skurray method also suggests that pair formation is not affected by membrane phase transition. The decline in pairing ability for cells of strain K1059 grown with either oleate or elaidate parallels that seen with pair formation as reflected by Coulter pairs. The differences in the numbers between Coulter and Skurray pairs probably reflects the difference in methodology (1) because determination of Skurray pairs requires additional dilutions and spreading of bacteria over agar surface which can disrupt loose pairs (i.e., specific pairs). Clearly the data further demonstrates that pair formation occurs with non-donor derivatives, but at frequencies lower than that observed with donor strains (Table VII).

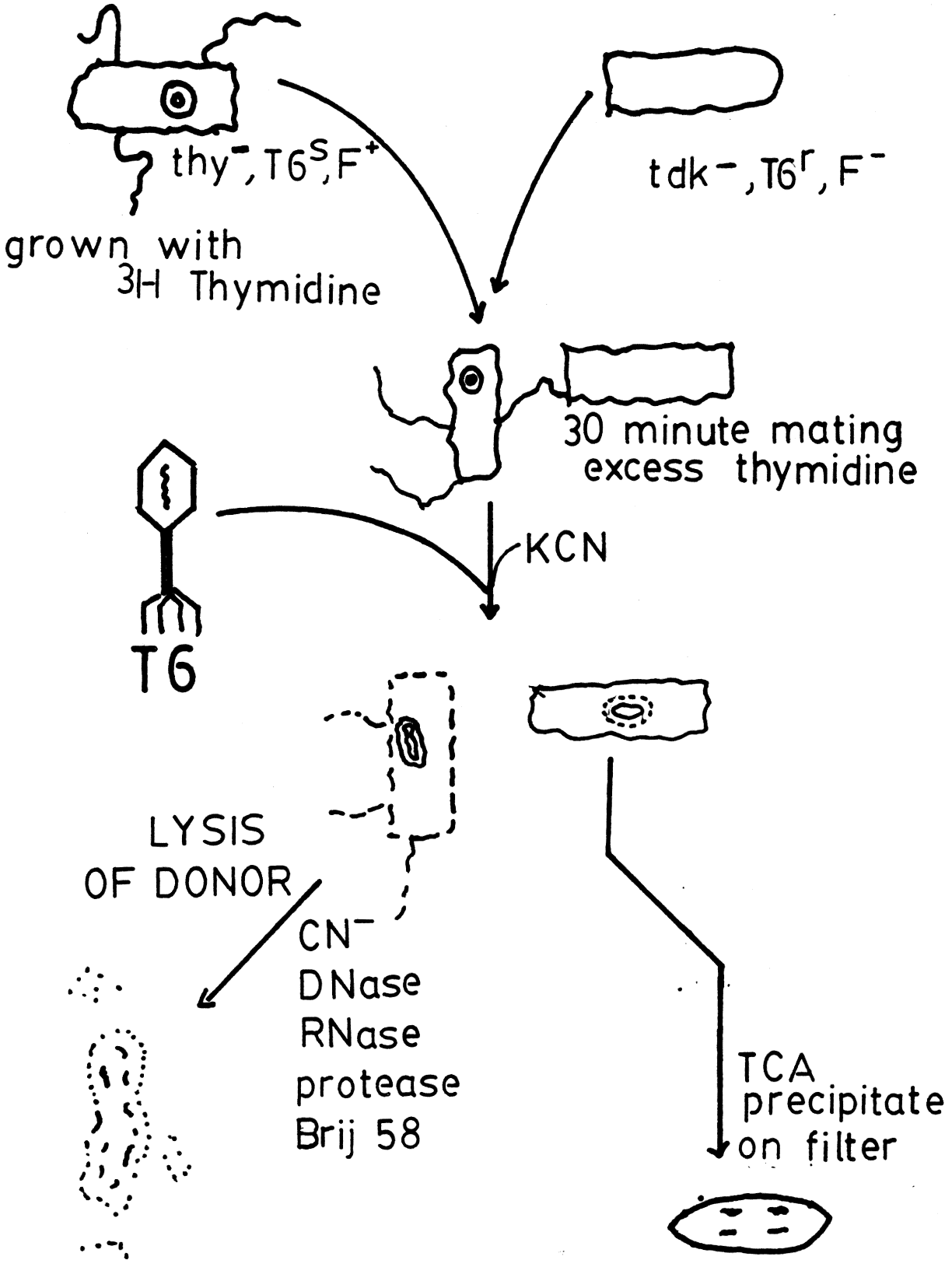
K. - Construction of Mating System for Measurement of Transfer of ^{3}H-thymidine labeled DNA.

Because neither pair formation nor plasmid maintenance appeared to be affected during membrane phase transition it was likely that one of the intermediate steps of conjugation, such as transfer of DNA from donor to recipient cell, was inhibited by an altered membrane state in the recipient cell. Measuring the amount of DNA transferred to the a recipient cell should indicate whether the membrane phase transition blocks the transfer step or (in the presence of normal transfer) one of the steps following transfer that are necessary for plasmid

establishment. In order to measure the transfer of DNA to recipient cells with ordered state membrane lipids, a mating system was required which simultaneously allowed selective removal of donor cells after transfer of radioactively-labeled DNA and the survival of Ufa$^-$ cells. The method of choice is based on a commonly used procedure that involves measuring the transfer of ^{3}H-thymidine-labeled DNA (from a thymidine-requiring donor) to a recipient cell incapable of utilizing thymidine (Tdk$^-$) (383). In this method, termination of the mating is accomplished by lysis of donor with the bacteriophage T6 (recipient is resistant, T6^R) followed by the degradation and solubilization of donor cell nucleic acids, proteins, and phospholipids, with DNase/RNase, protease, and detergent respectfully (169). Following lysis of donor cells, recipient cells are separated from solubilized donor cell components by centrifugation and washed with buffered medium. The recipient cells are then treated with 5% TCA to precipitate their DNA which is then collected on a membrane filter for determination of transferred-labeled DNA by scintillation counting (see Figure 34).

To ensure the utility of this system in matings with Ufa$^-$ auxotrophs, the following conditions had to be met. (1) The effect of the phase transition under mating conditions must not greatly affect the levels (by influx or efflux) of labeled-thymidine or labeled-products of thymidine metabolism in Ufa$^-$ auxotrophs grown with the different unsaturated fatty acids. (2) Ufa$^-$ strains must be shown to be capable of surviving the mating termination procedures in the absence of fatty acids and detergents. These components might possibly

Figure 34. Schematic Representation of Mating Procedure Utilized in Measuring Transfer of ^{3}H-thymidine Labeled DNA by Conjugation in Escherichia coli K12. Following growth of Thy^{-}, $T6^{s}$ donor in presence of ^{3}H-thymidine and growth of $T6^{r}$ recipient in label-free medium (MSCBO, MSCBE) cells are prepared for mating in the presence of excess cold thymidine. Following a mating period (0 or 30 minutes) cells are centrifuged and then suspended in Hershey-Chase adsorption medium containing T6 bacteriophage, KCN at non-lethal concentrations, tryptophan, thymidine, and deoxyguanosine. After a period of time allowing adsorption of bacteriophage and initiation of donor cell lysis, substances are added which will solubilize donor macromolecular fraction (DNase, RNase, protease, and Brij 58). Recipient cells, which remain, are centrifuged and washed in MS followed by precipitation with TCA (final concentration 5%). Samples are filtered, washed, and counted.


thy⁻, T6ˢ, F⁺
grown with
3H Thymidine
tdk⁻, T6ʳ, F⁻
30 minute mating
excess thymidine
KCN
T6
LYSIS
OF DONOR
CN⁻
DNase
RNase
protease
Brij 58
TCA
precipitate
on filter

inhibit T6 adsorption and the action of the degradative enzymes. (3) The donor cell must be shown to be lysed and their cell components solubilized by the mating termination procedures.

1. Ufa$^-$,Tdk$^-$ Recipient Utilization of Thymidine. A T6-resistant mutant (strain OT196) of strain K1059 was converted to Trp$^-$ by P1 transduction of Tn10::trp from Escherichia coli K-12 strain NK5151 and selection of tetracycline-resistant variants on tryptophan-supplemented medium. Tetracyline-resistant transductants were screened for tryptophan auxotrophy and one (strain OT197) isolated and purified. Strain OT197 was then transduced to Trp$^+$ by a P1 lysate of strain Escherichia coli K-12 strain KY895 and a Trp$^+$, Tdk$^-$ transductant (strain OT199) was detected by screening for resistance to fluoruracil in the presence of uracil and thymidine (168, 177). This Tdk$^-$ variant was still Ufa$^-$.

Although the Tdk$^-$ mutation prevents the conversion of thymidine to the DNA precursor, thymidine monophosphate, (168, 177) the mutation will not prevent some accumulation of thymidine when present in the medium because the transport function and other pathways of metabolism for which thymidine might serve as a substrate are not affected. Although unlikely, it is possible that a membrane phase transition may affect the levels of accumulation of free thymidine or its metabolized forms to such a degree as to alter the values obtained during measurement of transfer of labeled DNA. As a precautionary measure, accumulation of ^{3}H-thymidine by strain OT199 was determined under both growth conditions (confirming Tdk$^-$ phenotype) and mating conditions.

The data in Figure 35 demonstrates that strain OT199 is capable of accumulating only 10% of the thymidine relative to the parent mutant K1059, confirming its Tdk$^-$ phenotype, as indicated by resistance to fluorouracil. The data in Figures 36 and 37 indicates that regardless of whether cells are grown in MSCBE or MSCBO, the uptake of ^{3}H-thymidine is not affected in any peculiar way under mating conditions at either 39.5 C or 33.5 C. In addition, reversal of the temperature shift from 33.5 C to 39.5 C for either MSCBE- or MSCBO-grown cells does not result in any rapid efflux or influx of radioactivity (data point at 35 minute mark in Figures 36 and 37).

2. Selective Lysis of Thy$^-$ Donor. As one of the preliminary experiments for the eventual measurement of transfer of ^{3}H-thymidine-labeled DNA, the effectiveness of the T6-mating termination procedure on R100-1 donor, strain OT155, was determined. Addition of an equal volume of T6 bacteriophage in LB to a mid-log phase culture of OT155 in MSC-1% glycerol (multiplicity of infection (moi) equaled 10 phage per donor cell) resulted in approximately 5% survival of donors but the results were quite variable. Both low numbers of phage and the tendency of donor cells to clump under the conditions used to terminate the mating appeared responsible. Lengthening the period of T6 exposure (from 5 minutes to 15 minutes) did not reduce survival of strain OT155. Although logistically this is not a problem in plate matings, it does generate problems for measurements of transferred radioactively labeled plasmid DNA.

Figure 35. Confirmation of Tdk$^-$ Phenotype of Strain OT199. Strain OT199, a putative Tdk$^-$ variant of strain K1059 as determined by its resistance to fluorouracil, was tested for its ability to accumulate ^{3}H-thymidine into the cellular macromolecular fraction under normal conditions of growth. Cultures of strain K1059 and OT199 in early-logrithmic phase of growth at 39.5 C in MSCBO or MSCBE were introduced to 2 ug ^{3}H-thymidine/ml (20 mCi/mmol) and samples (100 ul) were subsequently removed for measure of ^{3}H-thymidine incorporation into TCA (5% final concentration) insoluble cell fraction. Samples were filtered over 0.22 um Millipore® membrane filters (25 mm dia) and washed with MS (4 C) containing 200 ug thymidine/ml and 10mM $Na_4P_2O_7$ for cultures of K1059 in MSCBO (■) or MSCBE (▲) and for cultures of strain OT199 in MSCBO (□) and MSCBE (△).

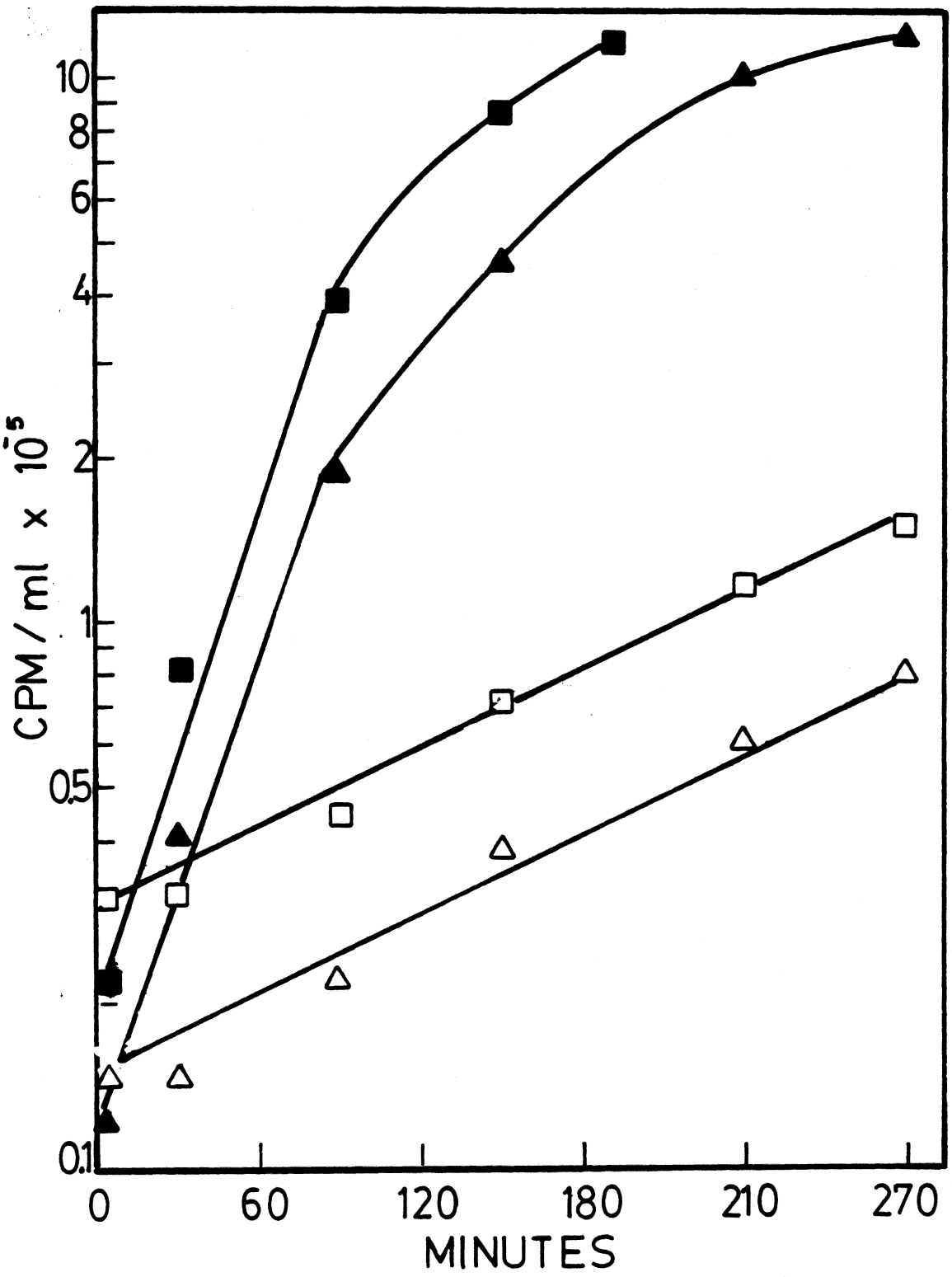

10
8
6
4
2
1
0.5
0.1
CPM / ml x 10⁻⁵
0
60
120
180
210
270
MINUTES

Figure 36. Uptake of ^{3}H-thymidine by Strain OT199 Under Mating Conditions Following Growth in MSCBO. Cultures of strains K1059 and OT199 in mid-logrithmic phase of growth at 39.5 C in MSCBO were prepared for mating by filtering and suspension in MS at 39.5 C. Cells were shifted to either 39.5 C or 33.5 C and the addition of the donor was simulated by the incorporation of an equal volume of MSC-1% glycerol containing 2 ug ^{3}H-thymidine/ml (20 mCi/mmol) and 200 ug deoxyguanosine/ml (39.5 C and 33.5 C respectfully). Accumulation of TCA precipitable counts (as described in Figure 32) was monitored for 30 minutes for all mixtures and for mixtures at 33.5 C an additional sample was taken 5 minutes following a reversal of the temperature shift back to 39.5 C. K1059 at 39.5 C (■), and 33.5 C (▲); OT199 at 39.5 C (□) and 33.5 C (△).

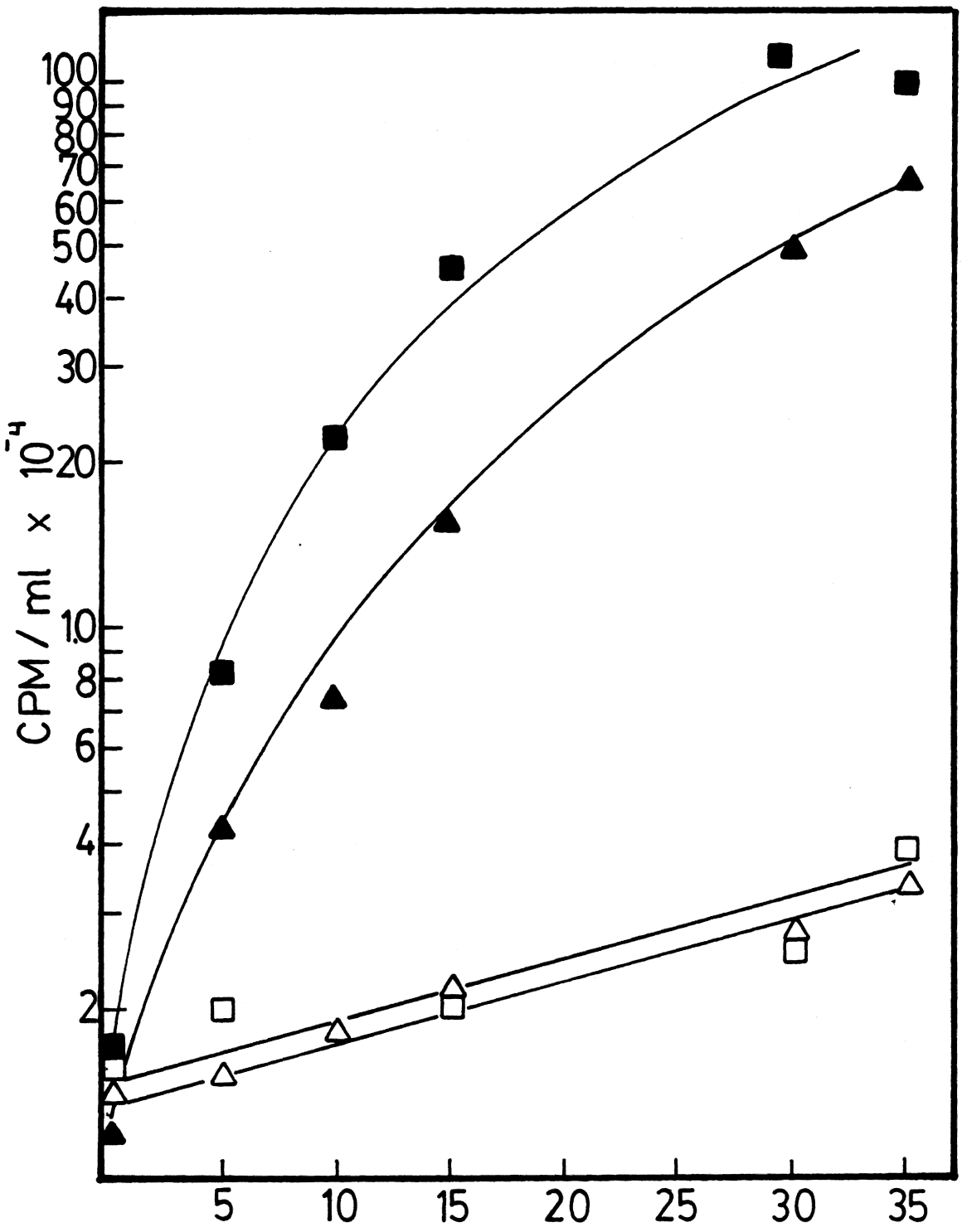
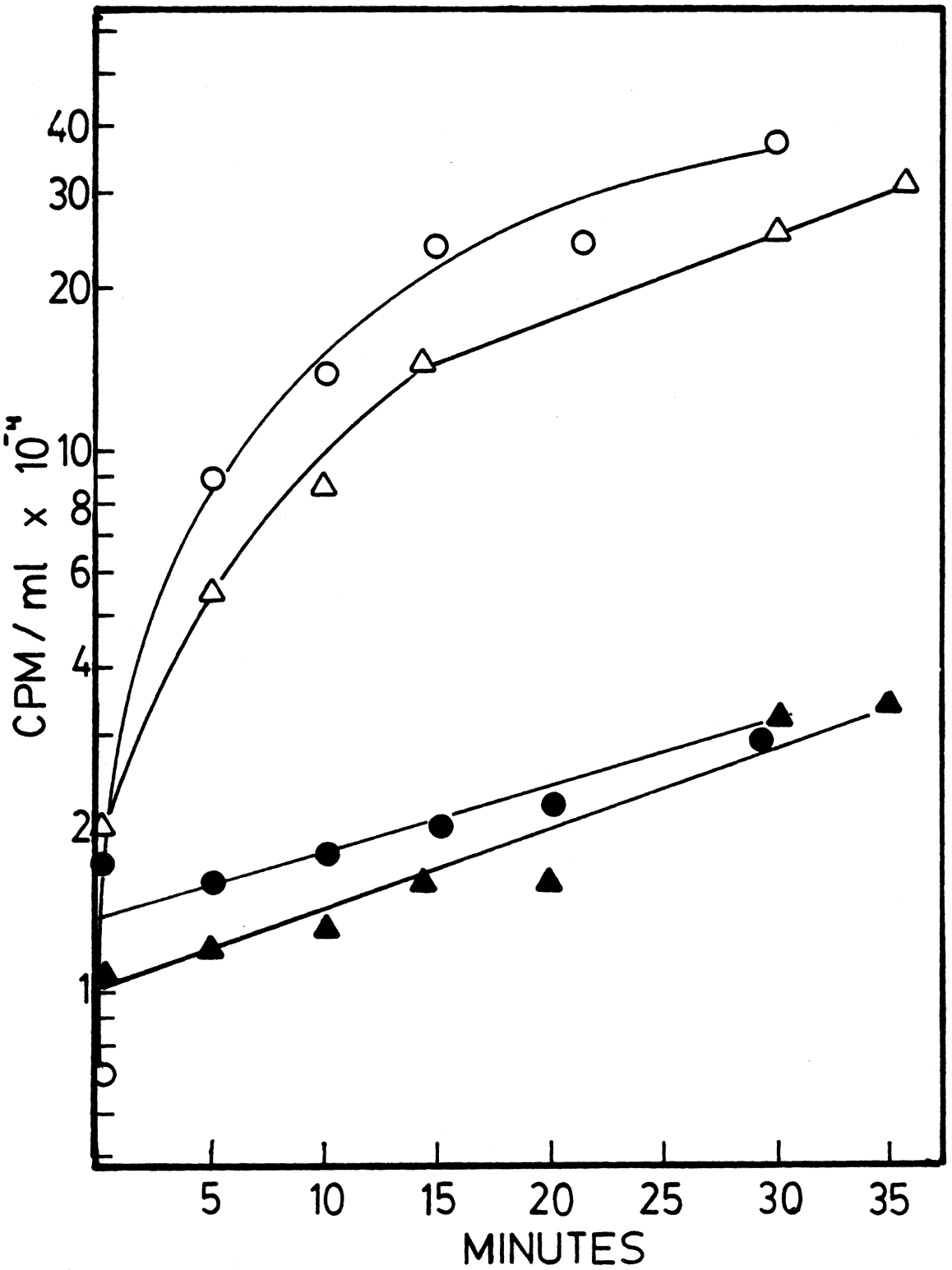

CPM / ml x 10^{-4}
100
90
80
70
60
50
40
30
20
10
8
6
4
2
5
10
15
20
25
30
35

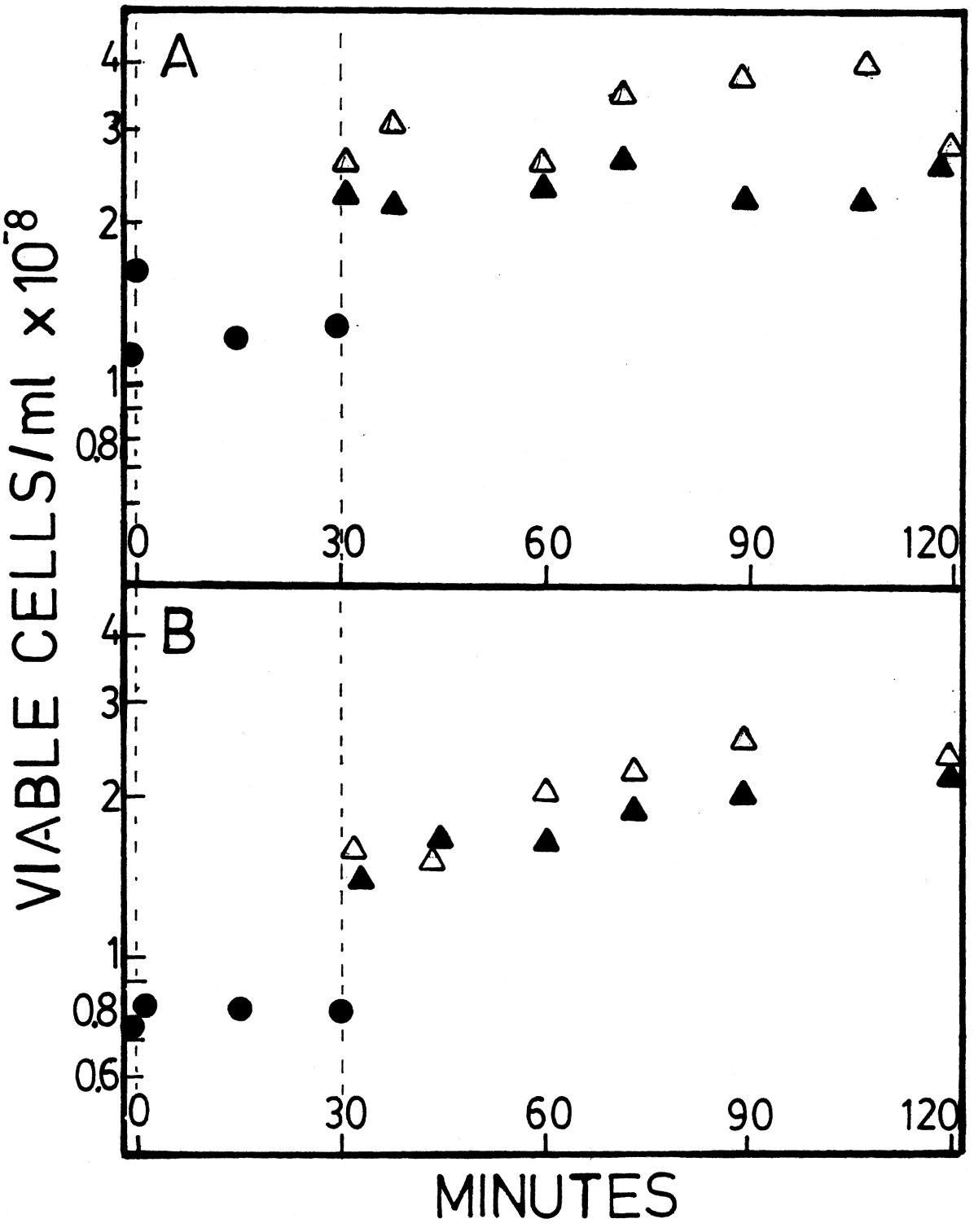
Figure 37. Uptake of ^{3}H-thymidine by Strain OT199 Under Mating Conditions Following Growth in MSCBE. Cultures of strains K1059 and OT199 in mid-logrithmic phase of growth at 39.5 C in MSCBE were prepared for mating by filtering and suspension in MS at 39.5 C. Cells were shifted to either 39.5 C or 33.5 C and the addition of the donor was simulated by the incorporation of an equal volume of MSC-1% glycerol containing 2 ug ^{3}H-thymidine/ml (20 mCi/mmol) and 200 ug deoxyguanosine/ml (39.5 C and 33.5 C respectfully). Accumulation of TCA precipitable counts (as described in Figure 32) was monitored for 30 minutes for all mixtures and for mixtures at 33.5 C an additional sample was taken 5 minutes following a reversal of the temperature shift back to 39.5 C. K1059 at 39.5 C (○), and 33.5 C (△); OT199 at 39.5 C (●) and 33.5 C (▲).


40
30
20
10
8
6
4
2
1
0
CPM / ml x 10⁻⁴
5
10
15
20
25
30
35
MINUTES

In an attempt to circumvent this problem, a mating termination medium which would allow a greater degree of killing of donor cells by bacteriophage T6 and allow survival of the recipient Ufa$^-$ strain was sought. The system developed involved collection of T6 in Luria broth in the usual manner (94) followed by precipitation of the T6 bacteriophage from Luria broth with 5% polyethylene glycol 600 (PEG 600) overnight at 4 C. Bacteriophage were then collected by centrifugation (12,000 x g for 20 minutes at 4 C) and then suspended in Hershey-Chase buffer (163) at approximately 2 x 10^{11} pfu/ml. Strain OT155 was found to be much more sensitive to attack by bacteriophage T6 (UV irradiated, 800 ergs/mm^2) when these cells were also suspended in Hershey-Chase buffer and subject to a high multiplicity of infection (moi) approaching 1000 (survival less then 0.01%). This medium was employed as the termination mixture upon addition of 0.01M KCN, 40ug tryptophan/ml, and 100ug thymidine/ml.

3. Survival of Strain OT199 Under Mating Termination Procedure. No problems were anticipated relative to the survival of OT199 in Hershey-Chase buffer containing 5 mM KCN based on the previous experiments (Figure 31, Tables IV, V). However, to confirm this supposition, MSCBO- and MSCBE-grown cells of strain OT199 were subjected to simulated matings using the new mating termination procedure. The data in Figure 38 and 39 demonstrate that strain OT199 could survive mating termination (> 99.5%) in Hershey-Chase medium following growth in either MSCBO or MSCBE and matings at either extremes of 33.5 C or 39.5 C whether matings are terminated at 0 minutes (Figure 38) or at 30 minutes (Figure 39).

Figure 38. Survival of OT199 Through the 30 Minute Mating Period and the Mating Termination Procedure. Cultures of strain OT199 in mid-logrithmic phase of growth at 39.5 C in either MSCBO or MSCBE were subjected to a simulated mating and mating termination procedures while cell viability was determined to allow detection of loss of recipients through the course of the experiment. Procedure is identical to that used in matings to determine transfer of ^{3}H-thymidine labeled DNA by conjugation (see Figure 35) with the exception of the presence of donor-labeled cells. Following initiation of mating termination procedures two samples of each mating mixture were simultaneously processed to measure reproducibility. (A) 39.5 C simulated mating for MSCBO grown cells (B) 33.5 C simulated mating for MSCBO grown cells (C) 39.5 C simulated mating for cells grown in MSCBE (D) 33.5 C simulated mating for cells grown in MSCBE.


A
B
VIABLE CELLS/ml x 10⁻⁸
MINUTES
4
3
2
1
0.8
0.6
0
30
60
90
120

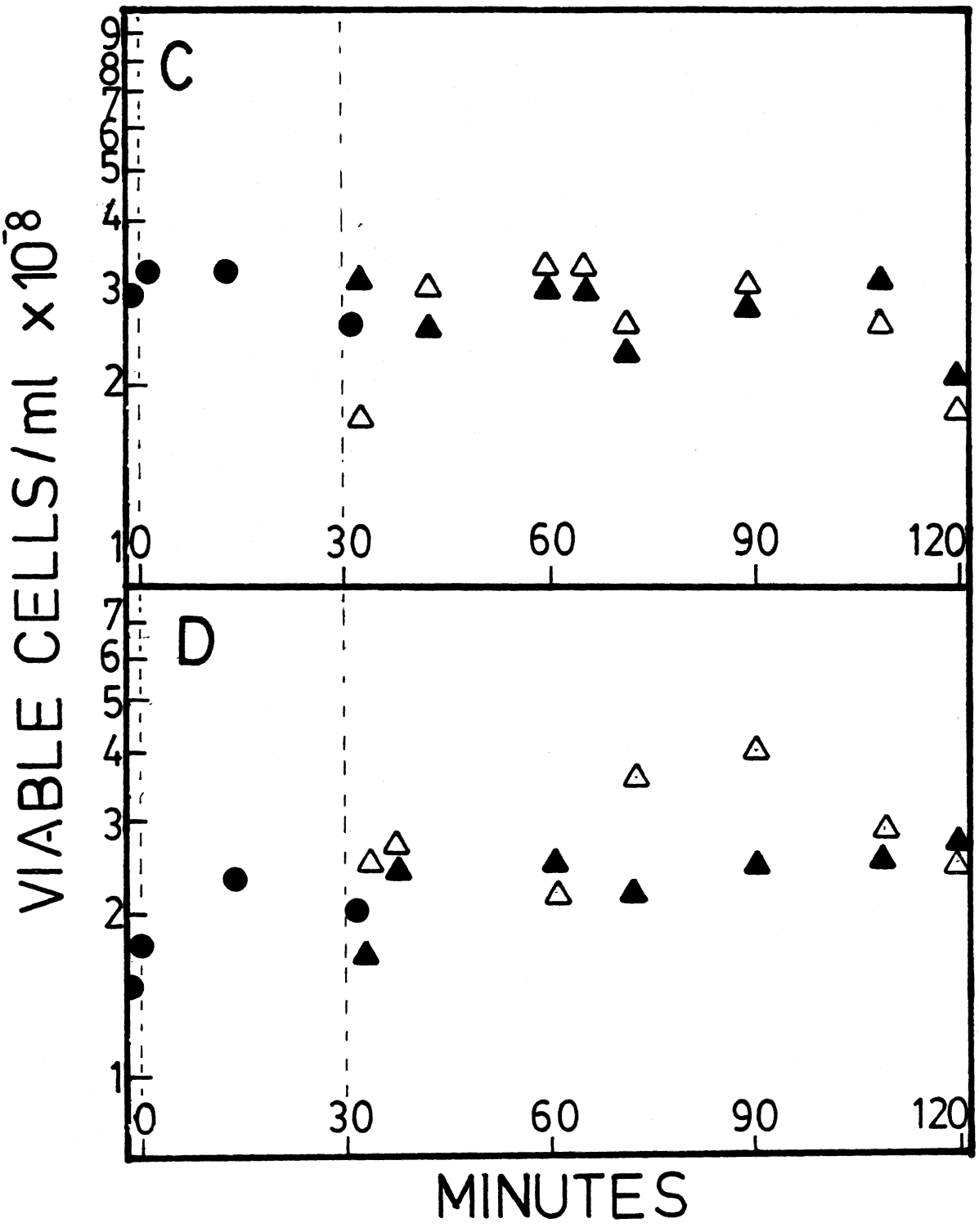
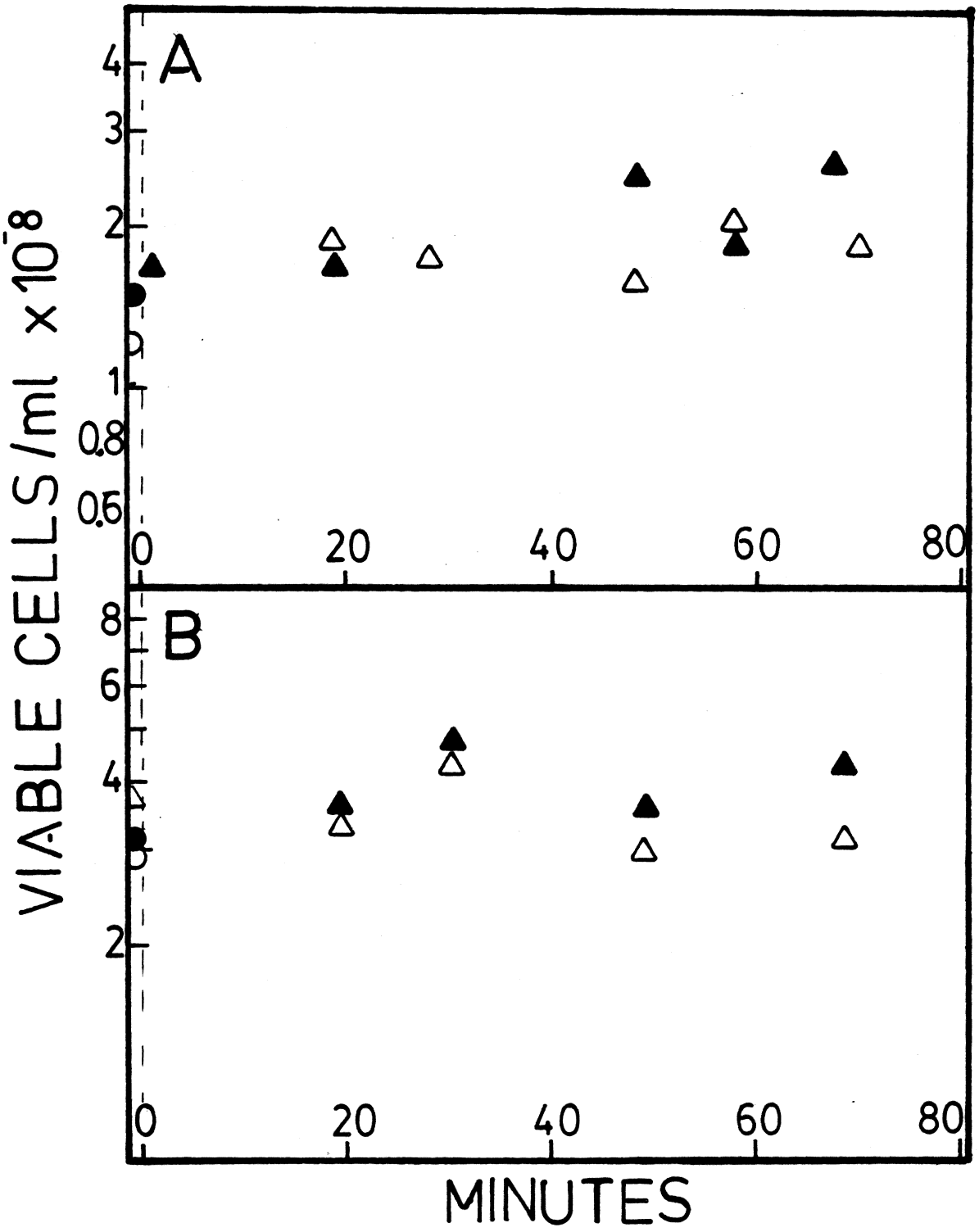
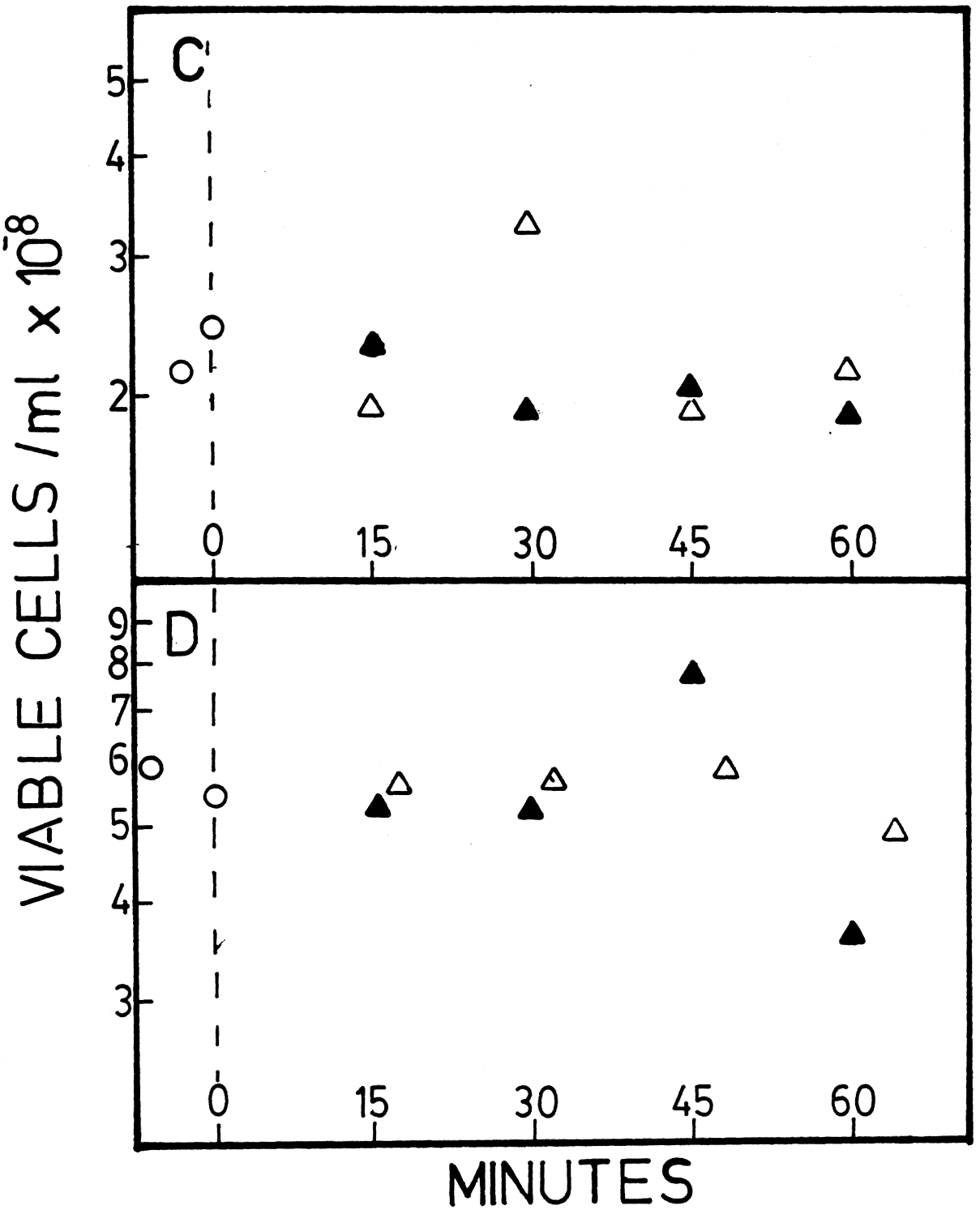

C
D
VIABLE CELLS/ml x10⁻⁸
MINUTES
0
30
60
90
120
1
2
3
4
5
6
7
8
9

Figure 39. Survival of OT199 Through the 0 Minute Length Mating Period and the Mating Termination Procedure. Cultures of strain OT199 in mid-logrithmic phase of growth at 39.5 C in either MSCBO or MSCBE were subjected to the initiation of a simulated mating and the mating termination procedures while cell viability was determined to allow detection of loss of recipient cells through the course of the experiment. Procedure was identical to that used in matings to determine transfer of ^{3}H-thymidine labeled DNA by conjugation (see Figure 35) with the exception of the presence of labeled-donor cells. Following initiation of mating, cells were immediatedly subjected to mating termination procedures where two samples of each mating mixture were simultaneously processed to measure reproducibility. (A) 39.5 C mating simulation for MSCBO grown cells (B) 33.5 C mating simulation for MSCBO grown cells (C) 39.5 C mating simulation for cells grown in MSCBE (D) 33.5 C mating simulation for cells grown in MSCBE


A
B
4
3
2
1
0.8
0.6
8
6
4
2
0
20
40
60
80
VIABLE CELLS/ml x10⁻⁸
MINUTES


C
D
VIABLE CELLS /ml x 10^8
MINUTES
0
15
30
45
60
2
3
4
5
6
7
8
9

L. Transfer of ^{3}H-Thymidine Labeled Plasmid to Cells Subject to Membrane Phase Transition.

Matings conducted to compare the level of transconjugants formed where the termination of matings was by either vortexing or by donor-selective lysis by T6 indicated that although a greater number of transconjugants arose from matings where T6 lysis of donors is employed, the same ratio of conjugation proficiency were maintained when comparing oleate- to elaidate-grown Ufa^{-} cells at different temperatures (Table V). This observation is expected in that it is known that a larger number of transconjugants will result if the recipient cells are allowed to incubate for a short period following DNA transfer prior to plating on selective medium (140, 183, 308). Using the mating system described above, the amount of labeled DNA transferred from donor strain OT155 to recipient strain OT199 was measured.

Matings were initiated by mixing ^{3}H-thymidine prelabeled donor and recipient cells in the presence of excess unlabeled thymidine (2000-fold excess over isotope). Samples of the mating mixture were subjected to the mating termination procedure immediately (time 0) and 30 minutes after the initiation of the matings. The results shown in Table VIII unexpectedly demonstrated that despite the reduction in the measured value for transconjugant formation of strain OT199 at 33.5 C following growth in MSCBE (> 98%), no relative difference in transfer of ^{3}H-thymidine labeled plasmid DNA was observed between MSCBO- and MSCBE-

Table VIII
Transfer of ^{3}H-thymidine Labeled R100-1 to Recipient Cells Subjected to a Membrane Phase Transition

Recipient Cell Supplement	Mating T^{o}	(a) Experiment	(b) Transferred CPM per 10^{7} donor CPM	(c) Transconjugants per 10^{5} donor
Elaidate	36.3 C	1	1.34×10^{4}	3.0×10^{4}
		2	2.55×10^{4}	N.D
Oleate	36.3 C	1	1.62×10^{4}	2.4×10^{4}
		2	2.78×10^{4}	N.D.
Elaidate	33.5 C	1	3.27×10^{3}	N.D.
		2	3.87×10^{3}	7.5×10^{2}
Oleate	33.5 C	1	5.6×10^{3}	N.D.
		2	5.1×10^{3}	8.3×10^{3}

Data from two experiments conducted as described in Materials and Methods. Cultures of strain OT155 were grown in the presence of 2 ug ^{3}H-thymidine per ml. (20 mCi/mmol) at either 36.3 C or 33.5 C. Cultures of strain OT199 were grown either in MSCBO or MSCBE at 39.5 C. All cells were prepared for mating upon reaching mid-logrithmic phase of growth (a). Following initiation of matings, samples were taken at 0 minutes and 30 minutes and processed as described in Materials and Methods and Figure 35. The quantity of DNA transferred was estimated from the difference in TCA insoluble counts between the two samples (b). Values were standardized by calculating transferred counts against the tritium-labeled TCA insoluble counts in donor (10 ul samples) at initiation of mating. Formation of transconjugants was determined by plate matings and standardized against donor cell number at initiation of mating as determined by viable count (d).

both elaidate- and oleate-grown cells received 25% of the labeled DNA transferred to controls at 36 C. Thus, ordered phase lipids in the recipient cell membrane did not prevent the R-plasmid donor cell from transferring DNA to some location interior to the outermost region of the cell envelope of the recipient cell.

DISCUSSION

A. The Results

Investigations of membrane phase transition phenomenon employing artificial phospholipid vesicles with reconstituted membrane lipids (115 ,223, 253, 282, 320) lysed cell vesicles (115, 216, 223, 253, 279, 283 320, 321) or, in some restricted cases, whole cells (104, 225, 281, 282 279, 294, 352, 360, 361, 391) have demonstrated that a temperature-induced change in membrane fluidity can simultaneously result in the alteration of the rate constant of many membrane-associated physiological processes. In concert with these observations, this investigation demonstrates that a disordered state membrane in recipient cells is required for optimal levels of transconjugant yields in matings with R100-1 plasmid donor cells. This observation was made possible through the development of a system which allowed the investigation of the effects of membrane fluidity changes in whole cells without the excessive loss in cell viability noted in other investigations (75, 249, 294). Extension of the basic system employed to demonstrate the effect of ordered phase membranes in recipient cells on transconjugant formation has allowed the initiation of investigations which will allow determination of the stage(s) of conjugation most affected by the altered membrane state.

Recipient ability, as measured by the formation of transconjugants, is altered in elaidate-grown Ufa$^-$ mutant cells of *Escherichia coli* when mated at temperatures known to reduce the fluidity of membrane lipids. The temperature below which a noticeable change in

transconjugant yield is observed (35 C) is in good agreement with physical determination of phase transition temperature in elaidate-grown Ufa$^-$ mutants using fluorescent probes (352). The 10-fold decrease in recipient ability of elaidate-grown Ufa$^-$ mutants within a 1.5C increment (35C-33.5C) relative to control matings of oleate-grown cells suggests a fairly extensive alteration in the function of the complex responsible for plasmid transport and establishment as required for completion of conjugation.

The observed reduction in recipient ability resulting from the alteration in membrane fluidity does not appear to be a reflection of any serious disruption in cell function or integrity. A membrane phase transition from disordered to ordered state for a period of thirty minutes and its reversal does not have any adverse effects on cell metabolism; both recipient ability and normal growth patterns (as measured by cell mass and colony forming units) return immediately with the reversal of the temperature-induced membrane phase transition. This absence of hysterisis with the reversal of the membrane phase state from ordered to disordered suggests that the effect of the temperature shift on conjugation proficiency is due solely to changes in the fluid state of membrane lipids. The paucity of experiments on the consequence of a reversal of phase transition on other physiological processes excludes any comparison between similarly based investigations. However, our results are consistent with electronmicrographic studies on phase transition and phase transition reversal in lipid bilayers (129, 216, 277, 320, 371, 372). Further

evidence suggesting that the effects of the membrane phase transition on cell integrity are minimal under the conditions used in the experiments reported here arise from the fact that the effect of the phase transition phenomenon on conjugation itself is limited, permitting normal function of many steps in the transfer process.

Despite a 10-fold decrease in recipient ability for elaidate-grown Ufa$^-$ mutants upon a temperature shift from 39.5C to 33.5C, the ability of these cells to form pairs at 33.5C is comparable to that of oleate-grown Ufa$^-$ mutants at the same temperature. The possibility that the union formation data is affected by non-specific cell agglutination is ruled out by the fact that the donor variant lacking the R-factor (strain OT100) forms pairs at much lower frequencies with either oleate- or elaidate-grown Ufa$^-$ mutants (Table V). Comparision of data on union formation generated in other investigations is difficult due to variability in pairing proficiency of mating cells arising from differences in cell pedigree, growth conditions, and duration of pair-formation period (374). However, unions as measured by Coulter Counter® generally occur within a range of 30 to 50% (374) with a maximum of near 70% (374) and generally their numbers are far in excess of the resulting transconjugant yields (374). The data presented in this work on union formation as measured by Coulter technique are comparable with these observations by other investigators. The discrepency between union formation as measured by Coulter technique and Skurray method likely arise from the potentially pair-disruptive manipulations required for completion of the Skurray

technique (1). Achtmann has found that the Skurray method may not allow detection of union formation with the same efficiency as the Coulter method; for example the Skurray method yielded a 30% lower value for union formation relative to the value derived from Coulter method in at least one instance (1).

Relative to the absence of measurable effects of membrane lipid phase transition on union formation, it appears that our data is consistent with results of other investigations. Curtiss and Stallions (99) have demonstrated that pairs form normally when energy metabolism is depressed or absent entirely. The presence of non-lethal concentrations of cyanide (CN^-) or the absence of a metabolizable energy source did not lower donor or recipient cell pairing ability (90). These results suggest that the interaction between pilus and receptor site is neither dependent on energy metabolism at other cell sites, de novo synthesis of structures requred for pair formation, nor assembly of a receptor complex. Pairing likely arises from an ionic interaction or an exergonic reaction for which energy is stored in the conformation of a structure (pilus) synthesized prior to the mixing of donor and recipient.

Orientation and structural integrity of the outer membrane receptor site is also expected to be maintained during the onset of a membrane phase transition because of the limited effect of a membrane phase transition on the outer membrane and particularly the outer leaflet of the outer membrane (277, 283, 351). In whole cells the outer membrane transition is not even detectable (247, 277, 351).

Comparison of isolated membrane fractions also suggest a very limited role for the outer membrane components in the phase transition event. In the isolated cytoplasmic membrane of Ufa^- mutants containing phospholipids with elaidate as the associated unsaturated fatty acid, (elaidate composing about 90% of the total fatty acid content), 60 to 80% of the hydrocarbon chains present will participate in the phase transition event (283, 320). In the isolated outer membrane of the same cell, only 25-40% of the hydrocarbon chains enter the ordered state (283, 320) and do so at a lower temperature than do the cytoplasmic membrane lipid components (283). The difference between the isolated outer membrane and the cytoplasmic membrane relative to phase transition behavior is apparently the result of differences in architecture, lipid to protein ratios, and the presence of lipopolysaccharides. The lipopolysaccharide of Ufa^- mutants contain only a minor amount of the unsaturated fatty acid added to the growth medium. Consequently, such cells maintain a fatty acid composition in the lipopolysaccharide which is comparable to that in wild type cells (e.g., saturated fatty acids of 12, 14, and 16 carbons, and β-hydroxymyristic acid (283, 351)). Although the saturated fatty acids associated with the lipopolysaccharide might normally participate in a phase transition event upon the lowering of temperature, the presence of the β-hydroxymyristic acid and the high protein to lipid ratio prevent the formation of a stable ordered phase (277). Additionaly, the location of all the lipopolysaccharide in the outer leaflet of the outer membrane may act to totally remove some

structures from the influence of the small areas of underlying ordered-phase phospholipids in the inner leaflet (277).

Investigations employing physiologically based-parameters also indicate an absence of disorder to order phase transition in the outer membrane. Colicin K is a bacteriocin that must adosrb to a specific outer membrane receptor site (294) and then become internalized by an undefined mechanism in order to generate its toxic effect on the host cell. At 10 C, a temperature below the T_h for phase transition in wild type cells (229, 294, 379), colicin K adsorbed to the wild type cell receptor site but did not result in the onset of cell death (229, 294, 379). Plate (294) extended this observation, demonstrating that elaidate-grown Ufa$^-$ mutants would adsorb colicin K at 31C but would not allow internalization of the colicin at this temperature. Specific to conjugation, the results reported in this investigation relative to union formation in Ufa$^-$ mutants suggest that the pilus receptor site in the outer membrane is not affected by the onset of a phase transition in cellular phospholipids. Furthermore, Curtiss has reported that union formation occurs at 2 C in wild type cells (91), a temperature below that known to cause phase transition in wild type cells of Escherichia coli K-12 (247).

Because pair formation was not altered by changes in the physical state of the membrane phospholipids, it appeared that the the stage of conjugation which is affected by a phase transtion was either that of transfer, establishment, or maintenance of the plasmid

following establishment. Since many low copy number plasmids (such as R100-1) appear to require some form of membrane attachment to effect maintenance (105,157,182), an alteration of membrane fluidity may disrupt the plasmids association with the membrane. However, our results on the stability of the plasmid-cell association during the onset and reversal of a temperature-induced membrane phase transition argue against this possibility. Using the Ufa$^-$ mutant carrying the R100-1 plasmid, colony-forming ability on media selective for plasmid-carrying cells was not reduced by a membrane phase transition. This experiment further demonstrates that plasmid-encoded resistance to either tetracycline or streptomycin is not dependent upon a disordered state membrane phase. Such a possibility might arise if the onset of a phase transition or its reversal resulted in the loss of cell membrane integrity, thus allowing an influx of antibiotics to a level exceeding the capacity of the cell to neutralize these compounds.

This experiment also addresses the possibility that a membrane phase transition or its reversal might cause disruption of plasmid structure, dissociation of resistance determinants from the plasmid occurring under some circumstances during transfer of the plasmid by conjugation (67). For R100-1, such an event might result in the dissociation of the tetracycline resistance determinant from the remainder of the R-factor resistance determinants (see Figure 35) as has been demonstrated for some R-factors following transfer by conjugation from Escherichia coli to Proteus mirabilis in (68, 263,

334). The results of the experiment on the effect of a membrane phase transition on the expression of resistance determinants indicate that a population of R100-1 carrying Ufa$^-$ cells do not demonstrate different levels of resistance to tetracycline and streptomycin upon the initiation of a membrane lipid phase transition to the ordered phase or its reversal suggesting that the plasmid structure is maintained intact However, because transfer of single-stranded DNA and establishment requires functions different from normal vegetative DNA replication, it is possible that the phase transition could result in selective loss of determinants only during transfer and establishment.

Inhibition of conjugation by a disorder to order phase transition in the membrane lipids of the recipient must arise from the disruption of one of the intermediary events in conjugation since neither the initial (pair formation) or terminal (maintenance) steps are affected. Experiments measuring the appearance of ^{3}H-thymidine-labeled plasmid DNA in recipient cells with ordered state membranes demonstrate that transfer is significantly higher than that expected on the basis of the observed conjugation-deficiency if only transfer was limiting. Transconjugant yield for elaidate-grown recipient cells at 33.5 C is only 3% of that for the same cells at 36.3 C (in agreement with earlier experiments) yet the amount of labeled DNA transferred to elaidate-grown recipient cells at 33.5 C for this mating was nearly 20% of that acquired by cells at 36.3 C. The amount of labeled DNA transferred to elaidate-grown cells at 33.5 C appears to be a significant quantity because oleate-grown Ufa$^-$ cells contained similar amounts of labeled

DNA in matings at 33.5C (about 24% of that for oleate-grown recipient recipients at 36.5C). The reduction in transconjugant yield for oleate-grown Ufa⁻ recipient cells between 36.3 C and 33.5 C (76%) parallels the reduced quantity of labeled DNA transferred indicating that the measured values of ^{3}H-thymidine labeled DNA transfer are reliable. Different values for accumulation of free ^{3}H-thymidine from the medium is not a potential source for misinterpretation because oleate- and elaidate-grown cells of Ufa⁻ mutants accumulate the same relative amounts of ^{3}H-thymidine at either 33.5 C or 39.5 C throughout the period of membrane phase transition and its reversal (Figure 35, 36). Furthermore, excess cold thymidine was present in the mating mixtures.

B. Model Development: An Explanation for Observations and the Direction of Further Investigation

1. Introduction. Using observations from this investigation and information available from the literature as parameters it should be possible to construct model systems which may be used as vectors for additional study on the effect of membrane phase transition on conjugation. Basically the models must offer explanations for the observations which indicate that DNA transferred to cells possessing ordered phase membrane lipids cannot be converted to the form of an established plasmid when transfer is completed and the phase transition is reversed permiting formation of disordered phase lipids. Offered below are two model systems which may explain the observations presented in this investigations. Information pertaining to this

study that was extracted from investigations in other laboratories is used to further refine the models.

2. Model I. Absence of Plasmid Establishment Following DNA Transfer to the Cytoplasm. During the event of DNA transfer by conjugation recipient cell functions appear to be as complex as those occurring within the donor cell (164). Thus, the intricate balance of RNA and/or protein synthesis (121, 122) juxtaposed with the initiation of DNA synthesis (311) in the donor cell may have a functional counterpart in the recipient cell. Among these putative recipient cell functions affecting the outcome of conjugation, there may exist some that are membrane associated and that are specifically required (or inhibitory) for establishment of transferred DNA although they may not be directly involved in the actual transfer process. If any of these functions were sensitive to the effects of a membrane phase transition it would be possible that DNA transferred to a recipient cell with ordered membrane lipids reaches the cytoplasm in a form prohibitive to its conversion to an established plasmid, even if phase transition was reversed after transfer. An observation by Falkinham and Curtiss (118, 119) which suggests a correlation between certain defects in membrane composition and an inability to stably inherit plasmid DNA adds support to the above contention.

Thus, it may be possible that DNA transfer occurs via some portal structure that remains stable in the presence of ordered phase membrane lipids and the membrane phase transition is instead inhibiting (or activating) the membrane associated function(s) of a process ancillary

to the transfer event but one which affects establishment. In the case where the membrane-associated function is one that is required for establishment, the model dictates that DNA transferred through the membrane remains sequestered from the membrane-associated functions which would permit establishment even with the reversal of the membrane phase transition. If the membrane associated function is one which, upon activation by the onset of a phase transition, prohibits plasmid establishment due to some modification of the transferred DNA, the reversal of the phase transition will have little additional consequence relative to plasmid establishment.

To test the assumption that DNA transferred to the recipient cells possessing altered membrane states is present within the cytoplasm in some form, a battery of physical and genetic techniques are available. Conversion of Ufa$^-$ mutants to minicell producing variants and the methodology of Skull et al. (329) may permit an anaylsis of the ability of the transferred DNA to associate with the recipient cell inner membrane when in the ordered state or after reversal to the disordered state. Isolation of plasmid DNA (64, 79, 206) from cells in possession of newly transferred DNA might also allow further insight into the step of conjugation inhibited by the onset of a phase transition. Density gradient centrifugation or use of single-stranded DNA specific nuclease (S1) in conjunction with DNA homology measurements might permit determination of whether transferred DNA was in the single-stranded or double-stranded form (64, 206). If the DNA found in the recipient cells was double stranded but not in a

covalently closed circular form, it might be possible that the DNA was subject to the action of a phosphatase. Treatment of isolated plasmid DNA with terminal deoxynucleotidyl transferase and DNA ligase should permit circularization if such is the case (395) and presence or absence of the CCC form of DNA can be detected through the use of density gradient centrifugation in the presence of ethidium bromide (79, 296). Finally, agarose gel electrophoresis and the Southern Plot technique (79, 111) may permit detection of plasmid DNA fragments if the plasmid was subject to restriction or exonuclease activity.

3. Model II. Entrapment of Transferred DNA in Periplasmic Space of Recipient Due to Phase Transition. The frequently used model of membrane fusion between mating cells would allow the free exchange of cytoplasmic components (91) and is thus obviously over simplified. Such an arrangement between mating cells would lead to excessive cell death upon artificial terminantion of matings be mechanical shear, an event which is not observed. It is apparent from recent investigations on lipid bilayer dynamics and cell morphology that lipid components of the membrane participate directly in the most complex of membrane functions simultaneous with their continued cell barrier functions. From these studies also arises a model for the direct involvement of membrane lipids in conjugation.

In this model, DNA transfer across the periplasmic space and the cytoplasmic membrane is dependent on the association of specific conjugation units (e.g. protein and/or LPS or phospholipid) with certain non-bilayer structures in the cytoplasmic membrane; possibly

the sites of association between the cytoplasmic membrane and the outer membrane commonly referred to as the zones of adhesion (25). The ordered phase state of membrane lipid would prevent the formation of non-bilayer membrane structures and disrupt existing ones. Consequently, the non-bilayer structure association with the conjugation transfer units would be destroyed. Under these circumstances, transfer of DNA to the cytoplasm is not possible but DNA transfer through the outer membrane into the periplasmic space may occur. Reversal of the membrane state to the disordered phase would allow formation of new non-bilayer structures, but DNA caught in the periplasmic space is excluded from the new zones of adhesion due to the inability to correctly associate with transfer units of conjugation if they are (at this point in time) even present at the zone of adhesion. DNA transfer to the cytoplasm under these circumstances would depend on low efficiency mechanisms of DNA internalization such as transformation/transfection. Support for this model and a means to determine its validity are presented below.

The existence of adhesion zones between the inner leaf of the outer membrane and the outer leaf of the cytoplasmic membrane (Figure 2) has been reported (17, 18, 22) and confirmed (80, 255). These zones of adhesion are involved in the transfer of LPS (21, 24, 255) capsule (26) and outer membrane proteins (338) to the outer membrane from their sites of synthesis at the cytoplasmic membrane. Observations which implicate a role for adhesion zones (or other non-bilayer structures) in conjugation include: (1) adhesion zones are the

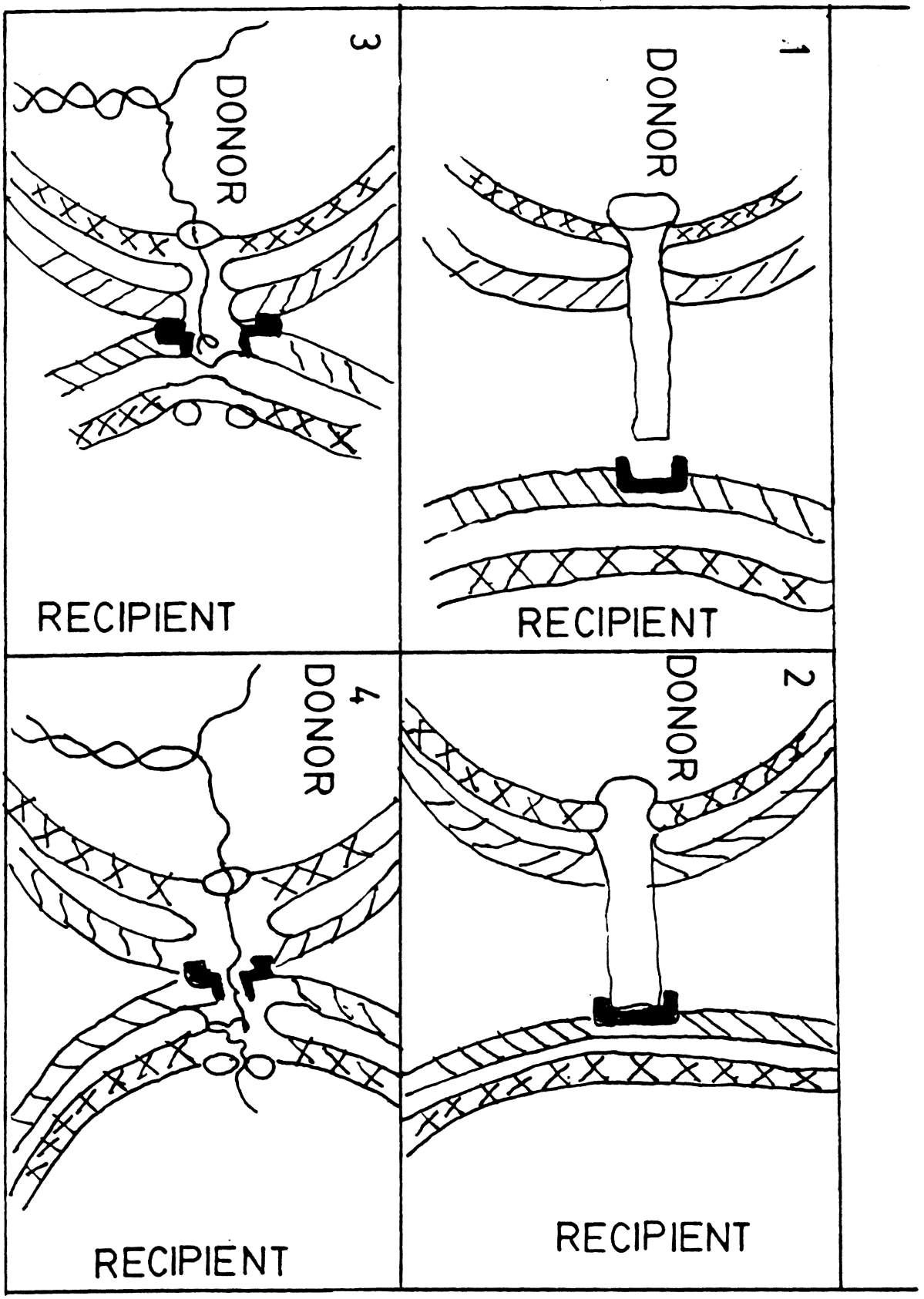
site of pilus insertion through the cell envelope of donor cells (23), (2) adhesion zones are the site of DNA injection of infecting bacteriophages such as K29, K26, T2, T1, T5, and BF23 (19, 22, 23), (3) certain molecules (such as the BF23/vitamin B12 receptor) possess functions in the area of the adhesion zones that they do not possess when they are removed (by diffussion) (16), and (4) stationary phase cells which lack adhesion zones (25) also are very poor recipients in conjugation (205, 353).

No specific mechanism for the assembly of adhesion zones has been suggested, though, the process involving a change in the membrane lipid structure from bilayer to non-bilayer phase as described in recent investigations on membrane fusion (40, 41, 81, 84, 367) may easily be pictured as the process involved. Current evidence indicates that membrane fusion events, in general, require the formation of non-bilayer phase lipids, such as the H_{II} form (81, 84) pictured in Figure 6. The apparent exclusion of normal outer membrane components from the zones of adhesion (20) may correlate with the requirement for protein free areas in the bilayer as a prerequisite to the bilayer to non-bilayer phase transition (84). The number and size of adhesion zones per cell 200-400 per cell at 200-300A^2 each; comprising about 5% of the total cell surface) (20) does not exclude the possibility that the adhesion zones are mostly protein free zones of non-bilayer lipid. Also, the proportion of phosphatidylethanolamine to phosphatidylglycerolphosphate found in natural membranes of Escherichia

coli closely approximates the minimal ratio of these two phospholipids required for the generation of isotropic phase lipids in artificial membrane systems (83, 301).

The proposed model for movement of DNA across the cell envelope may now be extended to the form schematically diagramed in Figure 40. Alignment of the pilus with the outer membrane receptor of the recipient cell triggers an event which causes localized disturbances in the inner leaf of the outer membrane and the outer leaf of the cytoplasmic membrane which lead to the association between an adhesion zone and the conjugation specific complex. Intra-cellular membrane fusion (inner with outer) resulting in the formation of an adhesion zone may even be a consequence of pilus association with the receptor. Bilayer stabilization by diphosphatidylglycerol (83, 301) can be eliminated by either introduction into the bilayer of free unsaturated fatty acids (84), phosphatidic acid (285), or by head group charge neutralization of the diphosphatidylglycerol with Ca^{++} influx (83, 301) (It might be appropriate to point out that transfection/ transformation normally requires an excess quantity of Ca^{++} (94, 232) possibly for similar reasons). Contact of recipient cell receptor site with the donor pilus could result in either activation of any of the nine known phospholipases of Escherichia coli (299), or the influx of divalent cations thus generating those conditions listed above that favor the formation of non-bilayer phase lipids in recipient cell membrane. Because of the low energy barrier between the two phases (85, 370), the conversion from bilayer to non-bilayer phase lipids may

Figure 40. Model for Trans-Membrane Transport of DNA by Conjugation. (1) donor cell pilus extending through outer membrane (/ / /) and the associating with the cytoplasmic membrane (X X X) in some fashion through an adhesion zone. makes contact with the recipient receptor site in the outer membrane. (2) pilus retraction bringing outer membranes of mating pair into during which time the pilus may slide over recipient surface making contact with receptor over an adhesion zone or possibly inducing conditions in recipient conducive for formation of adhesion zone in recipient. (3) receptor site is modified in some manner permitting initiation of transfer of DNA through to the recipient outer membrane region (4) formation of adhesion zone permits transfer of DNA from outer membrane region to cytoplasm through cytoplasmic membrane.


3
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occur without involving additional cell metabolism. Once the formation of the adhesion zone-conjugation unit complex is complete, DNA from the donor cell may be taken across the cell cytoplasmic membrane. This might be accomplished by the formation of some type of pore through the inner membrane or possibly by internalization of vesicle-like structures. This form of DNA uptake would avoid the consequences of any disruption in membrane continuity, but is difficult to correlate with the known linerally oriented entry of DNA transferred to recipient cells from Hfr donors.

The topic which remains to be confronted concerns the disorderd to ordered phase transition induced events that prevent passage of DNA across the cytoplasmic membrane, yet still allows passage over the outer membrane. In actuality, the basis for these criteria exist. Induction of a disordered to ordered phase transition occurs as the majority of phospholipid associated fatty acids come to assume an all trans conformation. Phosphatidylethanolamine and diphosphatidylglycerol possessing all trans fatty acids no longer can assume molecular conformations which favor the formation of non-bilayer phases (Figure 6). Since the generation of isotropic phase lipids is likely a prerequisite to the formation of adhesion zones, a disordered to ordered phase transition would prevent the generation of new adhesion zones and cause the disruption of existing ones. Obviously, the onset of a disordered to ordered phase transition would prevent the transfer of DNA across the cytoplasmic membrane if the zones of adhesion were required for completion of conjugation. Similarly, the disordered to

ordered phase transition induced accumulation of outer membrane proteins in the cytoplasmic membrane (104, 180) may also be a consequence of the absence of zones of adhesion through which outer membrane proteins must pass in reaching the outer membrane (338). The factors responsible for allowing passage of transferred DNA across the outer membrane but not the cytoplasmic membrane with the onset of a disordered to ordered membrane phase transition likely arise from the inherent differences in these two structures. The outer membrane lipids are induced into a disordered to ordered phase transition at lower temperatures (7C) than those of the cytoplasmic membrane (283) and only 25-40% of isolated outer membrane lipids participate in the transition (283, 320) and it is possible that no transition occurs at all in the outer membrane of whole cells (247, 277, 351). Furthermore, the outer membrane appears to be stablely oriented in the bilayer state, demonstrating no tendency to enter any isotropic phases (41).

The hypothesis that DNA transferred to cells possessing ordered phase membrane lipids is trapped in either the outer membrane or the periplasmic space might be confirmed with the following experiments. Following matings with donor cells grown in the presence of ^{3}H-thymidine Ufa^{-} mutant recipient cells could be subjected to either osmotic shock (10, 284) or converted to spheroplasts with lysozyme and EDTA in 20% sucrose in 0.1M Tris (pH 8.0) (323). Analysis of the supernatant from either preparation should allow detection of labeled DNA released from the periplasmic space while solubilization of the

cytoplasmic membrane (2% Triton X100) from lysed cells should allow detection of labeled DNA associated with the insoluble outer membrane.

C. Nature of Events Leading to Either Inhibition of Growth or Cell Death Upon the Initiation of Phase Transition in Whole Cell Membrane Lipids

An interesting finding of this work is that conditional cell death can occur upon initiation of a membrane phase transition. However, as also demonstrated in the dissertation, it is not a necessary consequence of the membrane phase transition. In regards to previous investigations on membrane phase transition phenomenon in whole cells where cell death may have led to misinterpretation of results (75), it would appear that extension of the observations arising from this study may lead to an improved characterization of the effects of membrane phase transition in whole cells. In particular, such investigations would allow the generation of additional methods for the determining of the effect of membrane phase transition on conjugation

It is obviously critical that the factors responsible for cell death upon the initiation of a membrane phase transition in Ufa$^-$ auxotrophs be identified such that they may be compensated for in additional studies. One observation reported here suggests a potential line of investigation regarding this matter. Elaidate-grown cells of the Ufa$^-$ mutants grown in glycerol demonstrate different capacities to survive temperature shifts in the presence of glucose, glycerol, or in the absence of a carbon source. Since the degree of the phase

transition should depend only on the physical state of the membrane at the initiation of the temperature shift, it would appear that the event causing cell death is secondary to the phase transition. Based on this premise, an investigation into the cause of cell death upon the initiation of a membrane phase transition should allow the development of variants of the Ufa$^-$ mutants which would allow their survival at low temperatures in the presence of various carbon sources.

To start the investigation it would be best to monitor major cellular functions at temperatures allowing survival and those that do not during the onset of a membrane phase transition. DNA and RNA synthesis could be monitored colorimetrically (diphenylamine for DNA and orcinol for RNA) or with isotope labeling (pulsed before the onset of phase transition). A similar approach utilizing combinations of gravimetric, colorimetric, and radiochemical techniques can be used to determine whether other systems are affected by the lipid phase transition (synthesis of proteins, phospholipids, peptidoglycan, and LPS). A second realm of investigation would center on the effect of a membrane phase transition on cellular energetics. Preliminary studies could incorporate the use of chemical reagents which specifically block electron flow or cause uncoupling of electron transport from ATP synthesis. Additionally, hemA$^-$ and Unc$^-$ variants of Ufa$^-$ mutants blocked in respiration and formation of functional ATPase respectfully, might also prove useful in this line of investigation.

SUMMARY

Evidence from other laboratories have suggested the possibility that conjugation in Escherichia coli may involve membrane fusion between mating cells (6, 94, 236) a process likely requiring complex changes in phospholipid composition and orientation (203). To test the hypothesis that membrane phospholipids might be involved in the process of conjugation it was determined from the literature that from among the available mutants altered in lipid metabolism the most information on the role of phospholipids in conjugation could be extracted from the use of the double mutant which can neither synthesize unsaturated fatty acids (fabB$^-$) nor degrade any form of fatty acids (fadE$^-$) (278, 330, 331, 332). Supplementing various unsaturated fatty acids to these mutants allow for initiation of conditions that allow alteration of the physical state and normal structural conformations of membrane phospholipids (75) a factor already found to affect numerous membrane associated functions (13, 108, 115, 129, 145, 223, 247, 253, 279, 283, 317).

The major obstacle in completion of the investigation of the effect of membrane lipid phase transition on the process of conjugation proved to be the loss of viability of the Ufa$^-$ mutant under the very conditions which allowed for changes in membrane fluidity. Although previously noted by some other investigators (64, 68, 75, 249, 294) the problem was most frequently ignored (75) or made inconsequential to the particular experiment (64, 68, 249, 294), neither condition adaptable in this investigation. Based on one investigation which

claimed cell viability could be maintained at 30 C for elaidate-grown cells of Ufa$^-$ auxotrophs we initiated our investigation by attempting to confirm this report. A discrepancy was noted between our results and those of the above cited investigators, apparently arising from the fact that the method of cell enumeration employed by the previous investigators (particle counter) does not discriminate between live and dead cells while our employment of colony forming ability as a measure of cell count only allows detection of normal healthy cells. Attempts to compensate for the factor which caused cell death at 30 C in elaidate-supplemented cells of strain K1059 led to the observation that cell death was not a direct consequence of the induction of a membrane phase transition but was secondary to this event likely arising from an imbalance of cell metabolism. The finding also indicated that the metabolic imbalance arose under those conditions which were required to allow successful levels of conjugation. Thus efforts were redirected towards defining conditions at higher temperatures which allowed the maintenance of cell viability in elaidate-grown cells subject to membrane phase transition. It was determined that these conditions could be met by suspending the elaidate-grown cells of Ufa$^-$ auxotrophs at temperatures between 33.5 C and 35 C in the presence of glycerol but not glucose. The effect of the membrane phase transition at these temperatures is readily reversible as is evidenced by the rapid increase in cell number and cell mass which occurs concomitant with a shift in temperature back to 39.5 C. This absence of hysterisis is consistent

with events observed in less complex systems where phase transition has been measured by physical techniques. Finally, the lambda lysogeny of strain K1059 has no measurable effect on cell viability during a phase transition and its reversal.

Based on these experiments, construction of the mating system was completed with the demonstration that Ufa$^-$ auxotrophs could survive various temperature shifts in the absence of unsaturated fatty acids and detergents (the absence of which is required for successful mating) for periods sufficient in length to allow measurable levels of conjugation. Following completion of numerous matings between an R-factor donor and the recipient strains K1059 and Ymel, the parent strain of K1059, it was apparent that a phase transition of membrane lipids did affect the level of transconjugant yield. Termination of mating by either mechanical shear or selective lysis of donor by T6 infection did not alter the ratio of transconjugant yield when comparing oleate- and elaidate-grown cells of strain K1059 at different temperatures of mating. The effect of the phase transition on conjugation is reversible.

The stage of conjugation most affected in recipient cells by the onset of a membrane phase transition appears to be an intermediate step between the time the recipient receives the DNA from the donor and the time when the plasmid is established. Particle counter measurements and Skurray pair analysis indicate that membrane lipid phase transition does not affect pair formation. Furthermore the measurement of ^{3}H-thymidine labeled plasmid DNA transferred to

the recipient cells in possession of altered membrane states also appears to be normal. Once established, the plasmid appears to be maintained in a normal state and is capable of directing neutralization of those antibiotics for which it possesses resistance determinants through the duration of a phase transition and its reversal.

The inability of cells with non-fluid membrane states to establish transferred plasmid DNA may be a function of such possibilities as; (1) failure to generate an established plasmid with that form of plasmid DNA which penetrates interior to the inner membrane (2) failure to successfully pass the transferred DNA from the outer membrane or periplasmic space into the cytoplasm.

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EFFECT OF TEMPERATURE-INDUCED MEMBRANE PHASE TRANSITION IN RECIPIENTS ON CONJUGATION IN *ESCHERICHIA COLI* K-12

by

Richard Thomas Smith

(ABSTRACT)

For the unsaturated fatty acid auxotroph (*fabB*$^-$) strain K1059, conditions have been found which allow for maintenance of viability following inhibition of cell growth by a temperature-induced membrane lipid phase transition. In matings with a F-like, R-factor donor, strain K1059 demonstrated a measurably reduced recipient ability at temperatures below 35.5 C when growth medium was supplemented with elaidate versus oleate. K1059 parent strain, Ymel, did not show variability in recipient ability when growth medium was supplemented with either of these two unsaturated fatty acids. Membrane phase transition induced inhibition of recipient cell function is reversible; elaidate-grown cells of strain K1059 held at a temperature of 33.5 C for 30 minutes demonstrating the same mating proficiency as cells remaining at the growth temperature of 39.5 C. Neither pair formation nor maintenance of plasmid once stably inherited appear to be affected by the onset of a membrane lipid phase transition. Furthermore, transfer of ^{3}H-thymidine labeled DNA from donor to recipient cell is only minimally affected by the change in recipient cell membrane lipid phase. Analysis of the results suggest that DNA transferred to recipient cells in possession of ordered phase membrane

lipids is either in a form which will not permit formation of a stable plasmid or is trapped in region outside of cytoplasmic membrane.