GROWTH CONDITIONS AND FREEZE/THAW SURVIVAL OF CHINESE HAMSTER OVARY CELLS

by

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ABSTRACT

The survival of CHO cells in tissue culture after freezing and thawing was assayed after altering the fatty acid constituents and the cholesterol content of the plasma membranes.

Only when cholesterol concentration has been increased in the membrane was survival altered at the slowest cooling rate of 1°C/minute. Survival at other cooling rates was unchanged.

Changing the fatty acid constituents in the membrane, as assayed biologically, had no effect on freeze/thaw survival. However, addition of avidin, a glycoprotein which binds biotin, significantly increased freeze/thaw survival at the slow cooling rate of 1°C/minute.

List of Tables

List of Figures

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INTRODUCTION

In 1970 Mazur published a paper including a graph in which cooling rate was plotted against freeze/thaw survival for various cells (Figure 1). These curves are distinguished by having one cell specific optimum cooling rate. The optimum cooling rate was interpreted to be the intersection of two adverse physical/chemical properties Mazur 1972). Damage at slow cooling rates was attributed to hypertonicity and damage at rapid rates to intracellular ice formation.

Various models have been postulated to account for injury at slow cooling rates Farrant (1973); Mazur (1970; 1972); Meryman (1975); Woolgar (1974); Lovelock (1955). Section II of the introduction is a review and critique of these models.

Fundamental to all the above models was the observation of one optimum cooling rate. Table 1 has been compiled as an aid to test the validity of this observation. Because many cell biologists do not include error bars on their freeze/thaw survival versus cooling rate graphs some of these points are open to dispute.

Damage from intracellular ice formation was thought to be curtailed by using a rapid thawing rate. Section III reviews the literature concerning this observation. There have been several reported anomolies and those are discussed in Section III.

Mazur (1972) suggested that the cell specific optimum was regulated by the permeability coefficient for water and the cell volume. Any change in either of these parameters should result in a different optimum cooling rate. Jain (1975) and De Gier (1967) reported any change in the unsaturated/saturated fatty acid ratio or cholesterol in the plasma membrane altered permeability. Both of these treatments were attempted in this thesis.

Choosing cholesterol had two added advantages. Increased membrane cholesterol decreased water permeability but also predisposed the cells to osmotic shock. Osmotic shock sensitivity was also assayed in this thesis.

Cholesterol changes were exclusive to the plasma membrane and the lysosome. Therefore, any change in freeze/thaw viability following cholesterol depletion or supplementation would implicate either of these two membranes Elias(1978).

The last part of this thesis is concerned with protection afforded by fetal calf serum. Section III reviews cryoprotection and specifically serum cryoprotection. Parts of this thesis attempted to ascertain the serum constituent responsible for cryoprotection. The counterpart of serum was thought to be egg yolk. Egg yolk protected against thermal shock as discussed in Section II under "Thermal Shock".

The anomolies discussed in Sections II and III of the introduction, the double optima, and the cell specific

nature of the cooling rate versus recovery curves suggested that there may be an additional variable to be considered. Section IV is a literature review of the biological damage as a function of cooling rate.

If damage were cumulative and non-specific, damage to cell organelles should be observed at all cooling rates. This would support a physical chemical approach to cryoinjury. If cell organelles were implicated in freeze/thaw injury, then any biochemical change that altered freeze/thaw survival should be manifested at all cooling rates slower or faster than the optimum. If the target varied with cooling rate, then a change in survival at one rate should be independent of survival at another. The review in Section IV suggests that the cell membrane is responsible for irreversible freeze/thaw injury.

This thesis is an attempt to introduce biochemical changes in the plasma membrane which would alter freeze/thaw survival. Biological assays were designed to specifically evaluate changes in the plasma membrane of Chinese hamster ovary cells.

Figure 1. Figure 9 from "Interactions of Cooling Rate, Warming Rate and Protective Additive on the Survival of Frozen Mammalian Cells" by Mazur et al. p. 82 in The Frozen Cell (A Ciba Foundations Symposium) 1978 edited by G.E.W. Wolstenholme and M. O'Connor J & A Churchill, London.

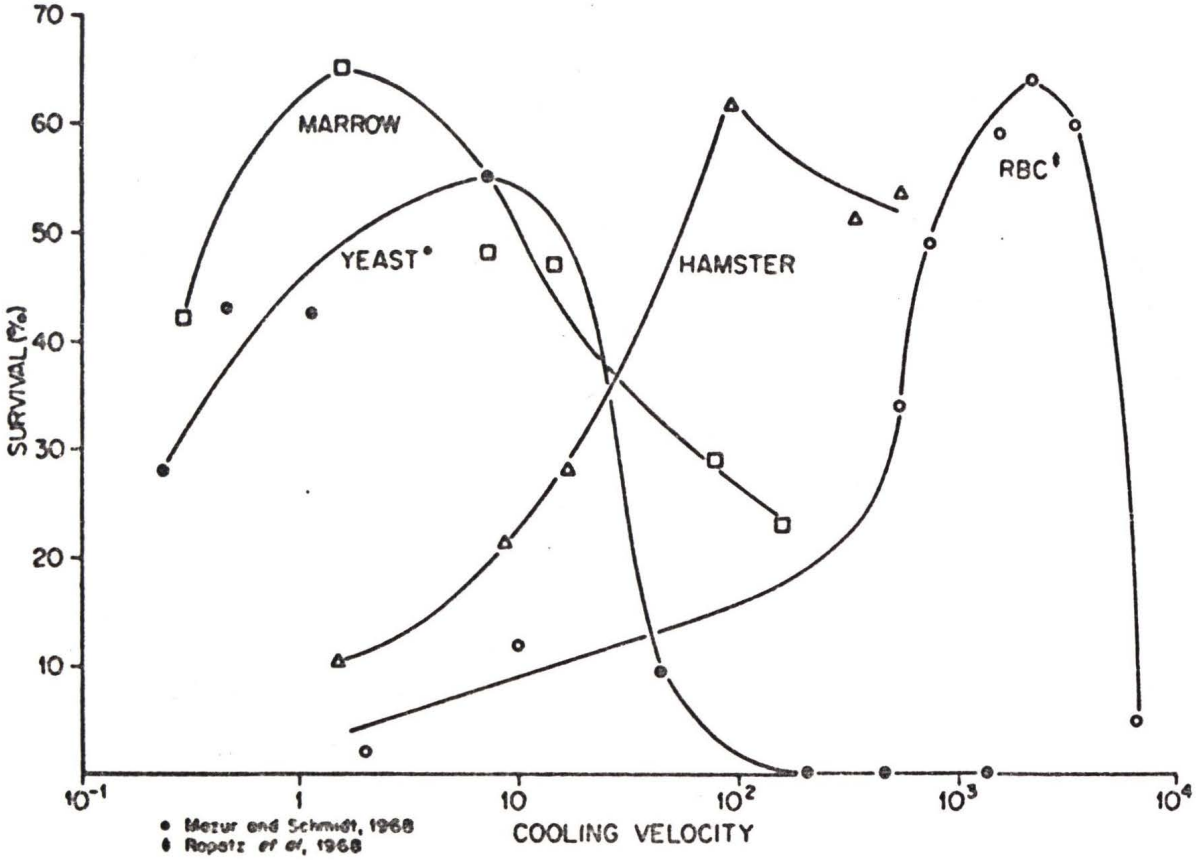

FIG. 9 Comparative effects of cooling velocity on the survival of various cells cooled to −196°C and thawed rapidly.

Optimum Cooling and Thawing Rates of Various Cell Types

CELL TYPE	CRYOPROTECTANT	COOLING RATE °C/minute	THAWING RATE °C
Escherichia coli		6 and 1000	
Streptococcus faecalis		10 and 10000	
Azobacter chroococcum		7 and 10000	
Pseudomonas aeruginosa		11 and 10000	
Klebsiella aerogenes		8 and 10000	
Salmonella typhimurium		10 and 10000	fast
P.H. Calcott (1974;1976)			
Saccharomyces cerevisiae		1 and 10	
P. Mazur and J. Schmidt (1968)			
anaerobic		10	
aerobic		5 and 20	
Neurospora conidia		10	
J.L. Leef and P. Mazur (1978)			
Acholeplasma laidlawii		1 and 9	fast
M. Raccah (1975)			
ERYTHROCYTES			
Human	0	1000	
	10% glycerol	500	
	10% DMSO	100	
	10% sucrose	1000	
	10% PVP	1000	
G.J. Morris and J. Farrant (1972)			
Miller and Mazur (1976)	1.3 M NaCl		

CELL TYPE	CRYOPROTECTANT	COOLING RATE °C/minute	THAWING RATE °C
Human	0	- 70 °C	
Bovine	0	- 120°C	
Ovine	0	- 140°C	
G. Rapatz and B. Luyet (1971)			
Bovine Spermatozoa	7% glycerol	40	7 seconds in 37°C
Cells			
human fibroblasts	10% DMSO	1 and 5	fast
D.T. Bodmer (1974)			
bone marrow cells	0.4 M glycerol	100	fast
	0.8 M glycerol	10	
	1.25 M glycerol	1-2	independent
	7.5% PVP	10	
	15.0% PVP	10	
S.P. Leibo (1970)			
platelets	12% DMSO	2	fast
J.P. Crowley, Anthony Rene, C. Robert Valeri (1975)			
rat hepacytes	10% DMSO	2-7 °C/minute	fast
A. Le Cam, A. Guillouzo, P. Freychet (1976)			
rat liver lysosomes	7.5% DMSO	10°C plateau	
J.A. Osborne, G.J. Morris, Donald Lee (197)			
Chinese hamster ovary	10% DMSO	10	100
L.W. Harris and J.B. Griffiths (1977)			
Chinese hamster lung	0.5 M DMSO	1 and 100	fast
fibroblasts	0.5 M glycerol	100	

CELL TYPE	CRYOPROTECTANT	COOLING RATE °C/minute	THAWING RATE °C
	0.5 M glycerol	100	
	0.7 M sucrose	100	very slow is toxic
	0.004 M PVP	100	"
P. Mazur (1972)			
Fetal rat pancreases	2 M DMSO	0.3	very slow
P. Mazur, J.A. Kemp, R.H. Miller (1976)			
human lymphocytes	10% DMSO	0.2 and 1	
	5% DMSO	0.1 and 5	
S.C. Knight, J. Farrant and G.J. Morris (1972)			
mouse lymphocytes	10% DMSO	1-3	∞
	5% DMSO	8-15	2.7 and greater
P.E. Thorpe, S.C. Knight, J. Farrant (1976)			
EMBRYOS			
rat embryos	1.5 M DMSO	0.8 to 1	5-24
D.J. Whittingham (1975)			
sheep embryos	1.5 M DMSO	0.3	12
S.M. Willadsen, C. Polge, L.E.A. Rowson, R.M. Moor (1976)			
rabbit embryos	1.6 M DMSO	1	5
H. Bank and R.R. Maurer (1974)			
mouse embryos	1.0 M DMSO	0.4	4-25
D.J. Whittingham (1972)			
sea urchin			
one cell embryo	1.5 M ethylene glycol	8	5 to 35
prism pluteus		4 to 25	10 to 70
unfertilized egg	no survival		
E. Asahina and Takahashi Tsueneo (1978)			

I PHYSICAL CHEMICAL FRAMEWORK

Mazur (1970), Farrant (1973), and Meryman (1975) have assumed that freeze/thaw cell survival was related to physical chemical factors of the extracellular solution. As the solution cools two phases appear, a liquid phase and a solid phase. As the solid fraction increases, the solutes in the remaining liquid phase become concentrated. The hypertonicity in the liquid phase is regulated by the original composition of the solution and the final subzero temperature. The initial concentration of the solute affects the volume but not the composition of the liquid phase found at any subzero temperature. Cooling cells must survive continuously increasing salt concentrations until the eutectic point is reached where only one phase remains, the solid one.

Ice crystal morphology in the solid phase can be manipulated by varying the cooling rate. Large crystals occur at slow rates and small crystals at faster ones. Very rapid cooling rates to liquid nitrogen temperatures (-196°C) can give rise to a supercooled state (vitrification) where no ice crystals are evident until thawing. If a fast cooling rate is followed by slow thawing, small ice crystals metomorphose into the larger ones (recrystallization). Cryoinjury is related to cooling and thawing rates. At slow rates, the hypertonic liquid fraction is the influence with which a cell must contend; at faster rates, the solid phase

is the external insult.

If freeze/thaw survival of cells merely assays the intensity of the hypertonicity of the liquid phase or the degree of intracellular ice formation in the solid phase, it would not be necessary to assess the nature of cell death. At slow cooling rates, the degree but not the nature of cell viability should vary until the optimum cooling velocity is reached. The nature of cell damage should not change until this point is reached.

If a cell were biologically manipulated to alter freeze/thaw survival at a slow cooling rate, then accordingly survival at all cooling rates less than optimum should be similarly altered.

II MODELS FOR FREEZING CELLS

The purpose of this section is to describe the various models that have been proposed to explain freeze/thaw survival. An attempt has also been made to present data which would dispute the credibility of each.

II A Lovelock's model

Lovelock (1953) correlated hypertonicity and freeze/thaw injury in erythrocytes. When slowly frozen cells and cells incubated in hypertonic solutions were compared, they responded by a volume decrease and an equivalent amount of hemolysis. Because these systems were sensitive to cation addition, in particular sodium, Lovelock (1953) attributed freeze/thaw damage to insidious effects of hypertonicity. He postulated four kinds of freeze/thaw damage: dilution shock, thermal shock, osmotic shock, and mechanical damage from intracellular ice crystals.

Dilution shock, significant during thawing, referred to the influx of water into the cell at high subzero temperatures. The dehydrated cell would be characterized by hypertonic intracellular ion concentrations. During thawing, cells would equilibrate with the extracellular liquid phase by admitting water. This is later discussed under the heading "Dilution shock" Woolgar (1974;1973); Pribor (1973;1974). Thermal shock described cryoinjury when cells, pre-treated with hypertonic solutions, were chilled but not frozen.

Farrant and Morris (1973) were concerned with this mechanism. Osmotic shock referred to damage resulting from slow cooling when cells equilibrate with extracellular hypertonicity by dehydrating. Osmotic shock is later discussed in sections dealing with "minimum cell volume" and "solution effects" by Meryman (1974) and Mazur (1970) respectively. Mechanical damage referred to the disruption of cell membrane integrity due to ice crystal formation. Ice crystal formation has been visualized by electron microscopy Bank (1973;1972); van Dujn Jr. (1974); Cruthers (1974); Nei (1975).

Lovelock's hypothesis which attributed damage to concentrated electrolyte solutions was discredited when Meryman (1971) discovered that solutions of equivalent osmolarities using sucrose and sorbitol, both cryoprotectants, produced similar amounts of hemolysis.

Secondly, decreasing electrolyte concentration in the extracellular solution 'might be expected' to alleviate osmotic shock damage found during slow cooling. When bacterial suspensions were frozen in isotonic salt solutions or in water, survival was much less in the former but the profile of the freeze/thaw curve was unaltered Calcott (1974).

II A-1 Cryoprotection

Lovelock (1955) found that glycerol was able to reduce the amount of hemolysis incurred by cooling but not chilling. Dimethylsulphoxide (DMSO) was also unable to prevent thermal shock damage Farrant (1972). Lovelock suggested that

cryoprotection conferred by glycerol was related to its intracellular permeability which would dilute intracellular electrolyte concentrations. Lovelock (1953), accordingly, adjusted the amount of intracellular glycerol by incubating cells at 25°C and 0°C. Cells with no intracellular glycerol succumbed to freeze/thaw, while those with glycerol survived. Mazur (1974;1976) suggested that inclusion of the copper ion in Lovelock's experiments may have drastically reduced the calculated amount of intracellular glycerol. His experiments suggested that survival was not related to cryoprotectant intracellular permeability. Similar results have been reported for mouse embryos and spermatozoa Leibo (1974); Bendston (1972).

Intracellular glycerol was not able to protect alone as was discovered in Escherichia coli and Chinese hamster ovary cells (CHO) Nath (1975); Taylor (1974). It was assumed, therefore, that protection was achieved by modifying the extracellular solution Mazur (1974).

Although much of what Lovelock embodied in his hypothesis was later to be disputed, he provided a framework for discussing the physical chemical aspects of cryoinjury.

II B Solution Effects

Mazur (1970;1972) proposed a model known as the "Two factor hypothesis".

In many cellular systems (Table 1), freeze/thaw survival versus cooling rate may be described by a parabola. The

summit or optimum cooling rate was presumed to be the intersection of two conflicting reciprocal forces. If survival were a function of time at a subzero temperature rather than cooling rate, then a slow cool/quick thaw regime should yield similar survival to a fast cool/slow thaw regime. Mazur (1972) found that fast cool/slow thaw resulted in much greater damage than slow cool/fast thaw. He surmised that cryoinjury at slow cooling rates was quite different from that found at faster rates.

He proposed the following model in 1970. As a cell was cooled, the cell interior was postulated to be supercooled. This created a difference between intracellular and extracellular vapour pressure. At fast cooling rates cells equilibrated by freezing intracellularly while at rates slower than the optimum, equilibrium was reached by dehydration. The optimum cooling velocity was interpreted as the rate where cryoinjury from both of these insults was minimized. Since this optimum was shown to be cell specific by Mazur (1970) he suggested that cell volume and permeability coefficient determined the rate.

Cholesterol depletion and unsaturated fatty acid changes in the plasma membrane of cells have been reported to alter the membrane fluidity and permeability coefficients in various cells Jain (1975); DeGier (1967). 'Increasing the permeability coefficient should predispose the cells to osmotic shock at faster cooling rates. The optimum cooling

rate should be shifted to higher velocities.'

Mazur (1977) predicted that increased cell volume should encourage a slower cooling rate. Data pertinent to this postulate were quite contradictory. Mouse embryos (70 um) and rabbit embryos (120 um) were frozen in 1 M DMSO and 1.5 M DMSO Bank and Maurer (1974). Rabbit embryos gave optimum survival at faster cooling rates. The difference between optimum velocities should be further emphasized had the same molarity of DMSO been used Mazur (1970).

The opposite relationship was exhibited by human, bovine, and ovine erythrocytes. The slowest optimum cooling rate was correlated with the largest cell mass Rapatz and Luyet (1971).

With the introduction of two-step cooling, Mazur (1977) suggested an additional variable, the nucleation temperature. In this system, Chinese hamster lung cells were incubated at a subzero temperature for an arbitrary time interval before plunging into liquid nitrogen McGann (1975). The subzero holding temperature was also vitally important. At holding temperatures exceeding -30°C, cells equilibrated with the extracellular solution by dehydration. Below this temperature equilibration was achieved by ice formation.

Chinese hamster lung cells frozen slowly in 0.5 M DMSO survived irrespective of thawing rate. Survival after quick cooling was contingent upon fast thawing Mazur (1972). Cells incubated at -25°C for 10 minutes before plunging into liquid nitrogen which would be equivalent to slow cooling,

demonstrate survival independent of thawing rate. Similarly cells incubated at -35°C for ten minutes, were susceptible to intracellular ice crystal formation and should be very sensitive to thawing rate. The opposite was true Farrant (1977).

Cells incubated at -35°C before plunging into liquid nitrogen, survived regardless of thawing rate used. The thawing rate was crucial when cells were pre-incubated at -25°C. Here slow thawing reduced survival to 1% from 70%.

Farrant (1977) suggested that the nucleation temperature for intracellular ice formation was -80°C, the highest subzero temperature where recovery was seriously impaired by slow thawing. Cells experienced dehydration effects down to this temperature although the rate of exosmosis would be retarded at these very low temperatures.

II B 1 Cryoprotection (Solution effects)

Mazur (1974) predicted that cryoprotection was not contingent on the intracellular permeation of DMSO or glycerol. Cryoprotection was related to altered vapour pressure by either increasing the external vapour pressure which would 'augment the volume of the liquid fraction' or by reducing the internal vapour pressure by encouraging dehydration.

'Vapour pressure of a liquid in an ideal solution should vary with the mole fraction of DMSO in solution. Since the composition of the liquid fraction containing DMSO is the same at any subzero temperature as predicted by phase diagrams,

the external vapour pressure* should also be identical at any subzero temperature. In my opinion freeze/thaw survival should not vary with initial concentration of cryoprotectant as reported by Mazur (1968) and Morris (1970).'

The postulate that cryoprotection was related to the colligative properties of cryoprotectants was contradicted by high survival of cells in less than the predicted colligative concentrations Meryman (1975). If cryoprotection were related to dehydration, high concentrations of DMSO or glycerol should confer a faster optimum cooling rate Mazur (1977).

Section II C which discusses the effect of increasing concentrations of cryoprotectants on optimum cooling rate demonstrates the opposite result. Mazur (1977) suggested that the augmented viscosity of these high concentrations increased the intracellular nucleation temperature by impeding the rate of cellular dehydration.

'If this were true, then one would expect at high concentrations of cryoprotectant, survival to be reduced owing to the increased residual cell water found at fast cooling rates.' Data supports the opposite supposition as shown by erythrocytes Morris and Farrant (1972); Pribor (1975); Tokio Nei (1976).

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* ed. Gordon M. Barrow, Physical Chemistry Third Edition McGraw-Hill Book Company New York 1973

Farrant (1977) presented another possibility. In two-step cooling cell response was regulated by two variables, the allotted time interval and the subzero temperature.

'If how cells equilibrate were related to the volume of liquid phase, then increasing the concentration of the cryoprotectant would be correlated with less dehydration, less shrinkage and a slower optimum cooling rates. With two-step cooling the amount of dehydration would be determined by the subzero temperature at which cells were incubated. Increasing the percentage of DMSO would serve to decrease the optimum bath temperature or to increase the amount of time necessary at a subzero temperature.'

In summary, Mazur (1977) suggested that as a cell cooled, its intracellular constituents became a supercooled liquid while the external environment exhibited two phases. The difference in vapour pressure between the inside and outside of a cell was resolved in two completely different ways. Cells cooled very slow rates, dehydrated. At faster cooling rates cells equilibrated by freezing intracellularly. The number of effective nucleation sites varied directly with the cooling rate. The size of the resulting ice crystals were circumscribed by the cooling and thawing rate. Upon thawing recrystallization occurred at -25°C Mazur (1972).

'It would be interesting to see if those cells containing intracellular glycerol and a vapour pressure equivalent to the external solution, exhibit a much slower optimum cooling

rate than those cells lacking glycerol. Cells without glycerol permeation would be expected to dehydrate and be damaged.'

II C Minimum Cell Volume

Mazur and Meryman have discussed the physical events responsible for cell death during freeze/thaw. Mazur believed that these events were related to dehydration and Meryman, to minimum cell volume.

Meryman (1971) proposed this model after completing a series of experiments using potassium-depleted erythrocytes. Sensitivity to osmotic shock, thermal shock, and freeze/thaw damage was correlated closely to cell volume rather than intracellular electrolyte concentrations. Survival was related to a crucial minimum cell volume. Compelling a cell to shrink beyond this volume would induce reversible membrane leaks; reversible since viable frozen/thawed erythrocytes contained high sodium and low potassium concentrations when compared to unfrozen controls. Should freeze/thaw facilitate the entry of normally impermeable solutes, dilution and post-hypertonic shock would ensue with thawing.' Mazur (1970) predicted that damage due to hypertonic shock would be alleviated by slow thawing or by incubating in slightly hypertonic post-thaw solutions.

The second assumption, implicit to this model, was that cryoprotection was related to colligative properties. Meryman (1977) argued that cell death could be correlated

with hypertonicity regardless of subzero intracellular cryoprotectant concentration or temperature. Because cryoprotection was contingent upon cellular infiltration, the intracellular osmolarity would approximate that found extracellularly which would reduce the degree of shrinkage needed to achieve equilibrium.

Where protection was mediated extracellularly osmolarity was postulated to be the relevant parameter. That is, this osmolarity initiated cell dehydration until reaching a minimum cell volume where hemolysis ensued. Hemolysis in presence of extracellular protectants was correlated with extracellular osmolarity, not the hypertonicity. Where freeze/thaw was conducted using extracellular cryoprotectants Meryman advised faster cooling velocities for optimum recovery Meryman (1977).

Meryman (1971) speculated that upon reaching the minimum cell volume, large extracellular molecules would be admitted into the cell. Protection would be achieved by decreasing the amount of shrinkage by increasing intracellular osmolarity as with glycerol or DMSO. Both glycerol and DMSO were defined as penetrating cryoprotectants. There are several problems with this model. Meryman (1977) predicted that extracellular cryoprotectants would favour a faster cooling velocity as was observed in bone marrow cells and Chinese hamster lung fibroblasts with polyvinylpyrrolidone (PVP) Leibo (1970); Mazur (1972). An anomoly, however, was presented by sucrose,

a non-permeating cryoprotectant, which exhibited the same survival/cooling rate profile as glycerol and DMSO, permeating cryoprotectants Leibo (1970).

Very high concentrations of PVP, dextran, sucrose, and bovine serum albumin behaved much like permeating agents during freeze/thaw Leibo (1970). One might expect this if membrane stress were proportional to extracellular cryoprotectant concentrations. Should membrane stress be relieved by leaks which admitted PVP and sucrose a residue of these chemicals would be detected following freeze/thaw and fundamental to cell survival.

The difference between permeating and non-permeating agents was not clearly established experimentally. (Section III C in this thesis) There are three approaches to these data. First permeability and freeze/thaw survival might be independent variables; secondly the ability of a cryoprotectant to permeate a cell might impose additional damage during freezing and thawing Woolgar (1974); or thirdly, infiltration of any cryoprotectant might be vital to freeze/thaw recovery Meryman (1971).

The last alternative was shown to be unlikely when Daw (1973) discovered that although sucrose was admitted into the cell during freeze/thaw, it was indicative of cell death. This would suggest that leaks were correlated with hemolysis. Secondly, post-hypertonic shock should be maximized at slow cooling rates in high concentrations of extracellular agents.

The opposite behaviour was observed in erythrocytes by Pribor (1973) and Morris (1972). Survival approaches 100% at concentrations conjectured to produce the greatest post-hypertonic and dilution shock Pribor (1976); Nei (1975); Mazur (1974); Morris (1970).

Mazur (1974;1975) has speculated about the relevance of intracellular permeation. Glycerol mutants of Escherichia coli exhibited no correlation between freeze/thaw survival and permeability Nath (1975). This was also found to be true of bovine erythrocytes in glycerol Mazur (1974); of spermatozoa in glycerol Wiggin (1975); and mouse embryos in DMSO Leibo (1974).

Although erythrocytes did not exhibit increased survival with glycerol permeation, they did experience a temporary increased sensitivity to freeze/thaw after a few minutes. Increasing glycerol concentrations delayed the time at which this dip appeared. Sucrose incubation did not demonstrate the same behavior Mazur (1974).

Glycerol (1M) suspensions of erythrocytes diluted to 6 M glycerol at 0°C, simulated this temporary decrease in freeze/thaw survival Leibo (1976). This dip might be related to thermal shock.

If "minimum cell volume" accounted for cryoinjury sensitivity to freeze/thaw should coincide with minimum cell volume. This volume was achieved immediately upon cryoprotectant addition as demonstrated in bovine erythrocytes

Mazur (1974), and CHO cells Mironescu (1978). However, human erythrocyte behavior was consistent with this expectation Mazur (1976).

This observed dip in freezing resistance of bovine erythrocytes might coincide with a shift in the optimum cooling velocity. These data were collected using only one cooling rate, slower than optimum.

A summary of Meryman's model was as follows. Osmolarity and hypertonicity were the physical factors with which a cell must contend during freezing and thawing. High osmolarity resulted in membrane stress which could be alleviated either by admitting an intracellular cryoprotectant which would increase intracellular osmolarity, or by cooling at faster rates in the presence of extracellular cryoprotectants which would retard dehydration due to viscosity in the liquid phase Meryman (1975;1978).

II D Cold Shock

Cold shock or thermal shock resulted when erythrocytes were exposed to hypertonic solutions of salt at 25°C before chilling to pre-freezing temperatures Lovelock (1953); Meryman (1972).

Susceptibility to thermal shock was proportional to pre-chilling hypertonicity. Farrant (1973) later investigated thermal shock in erythrocytes and suggested as Lovelock (1953) did, that similar events were experienced by a cell during freeze/thaw. Survival of cells in hypertonic

solutions of sucrose and sodium chloride at 0°C were compared to those undergoing thermal shock or freezing and thawing. Cell mass, sodium and potassium exchange, hemoglobin release, and hemolysis were assayed Farrant (1972;1972).

Both hemoglobin concentrations and cell shrinkage reached a plateau when osmolarity of the solution at 0°C exceeded 1500 milli-osmoles. Hemolysis in hypertonic solutions occurred at 2000 milli-osmole, the same osmolarity where high intracellular concentrations of electrolytes were suddenly alleviated. Leaks were postulated since both intracellular hemoglobin concentrations and cell volume remained constant above 1500 milli-osmole. These leaks were selective in hypertonic solutions regardless of composition since no labelled sucrose was detected intracellularly Farrant (1972).

Sucrose, which protected against hemolysis at osmolarities exceeding 1000 m osmoles, parallelled these events insofar as cell mass reduction and potassium depletion were concerned. Intracellular sodium chloride influx was not as pronounced as in salt solutions of equivalent osmolarity. Farrant (1972) concluded that hypertonic hemolysis was related to these leaks, a function of the extracellular osmolarity. Protection from sucrose was attributed to the dilution of extracellular electrolyte concentrations which would limit the intensity of electrolyte invation.

How does hypertonic shock hemolysis compare to thermal

shock hemolysis? Osmolarity was the measured parameter. Sucrose solutions had a lower electrolyte concentration than salt solutions of equivalent osmolarity. Cells suspended in hypertonic sucrose solutions demonstrated resistence to thermal and hypertonic shock Morris (1973).

Damage due to chilling from 25°C to 0°C was more pronounced than hemolysis of erythrocytes in hypertonic solutions at 0°C or 25°C. Times of exposure were identical. Chilling either impeded the cell's ability to surmount hypertonic shock as was suggested by Morris (1973) or imposed an additional stress unrelated to hypertonicity.

How then does thermal shock alter cell mass, electrolyte exchange, and intracellular water loss? The onset of hypertonic shock hemolysis at 0°C was observed in salt solutions of 2000 milli-osmoles. Survival in sucrose solutions of equivalent osmolarity was 100% Farrant (1972). Thermal shock hemolysis, however, was manifested at 1000 milli-osmoles and 1500 milli-osmoles in salt and sucrose solutions respectively Morris (1973). Thermal shock hemolysis was also peculiar in its admission of labelled surcrose or albumin in both sucrose and salt solutions Daw (1973).

If the "leak" phenomenon sensitized cells to thermal shock, survival of erythrocytes should be related to the amount of intracellular electrolyte depletion, macromolecule infiltration or cell water content. While thermal shock survival in hypertonic salt solutions supported this

postulation the sucrose datum contradicted it. Chilling in sucrose did not deter electrolyte exchange, intracellular water loss or changes in cell mass. The sucrose control also predisposed the cells to leaks which admitted macromolecules Daw (1973).

If thermal shock survival were a function of "reversible leaks" which relieved cell membrane stress incurred during shrinkage, there should be a positive correlation between recovery and sucrose infiltration. Daw (1973) found that thermal shock hemolysis was correlated with macromolecule leaks. Even the claim that exposure time determined the intensity of sucrose invasion and therefore the degree of thermal shock hemolysis was disputed. Survival in hypertonic salt solutions with respect to cooling velocity had two optima Morris (1973).

If thermal shock were related to cell volume reduction, thermal shock recovery should be optimized where cell volume approximated that of the control in salt solutions. In sucrose, recovery would be a function of sucrose invasion and a larger cell volume. This prediction was confirmed in hypertonic salt solutions. Recovery in sucrose was not correlated with cell volume at all. Recovery was least when cells had an intermediate cell volume and intracellular water mass Daw (1973).

Farrant (1972) also found that DMSO, a cryoprotectant, was unable to mitigate the severity of thermal shock although it alleviated cell volume reduction and electrolyte exchange.

This would suggest that these parameters may have no relationship to thermal shock survival.

Morris (1975) also postulated that thermal shock was not related to hypertonic shock. He discovered that thermal shock did not augment cellular phospholipid and cholesterol in the supernatent as was observed during hypertonic shock treatments by Lovelock (1954;1955).

How does thermal shock relate to events occuring during freezing and thawing? If thermal shock were to simulate the sequence of events undergone during freezing and thawing, its rigour should be modified by varying cooling velocity Morris (1973).

Cooling velocity was nominally significant in thermal shock treatment. Cells, pre-incubated in hypertonic salt solutions, exhibited damage with fast cooling rates and those incubated in sucrose solution were susceptible to slow cooling rates.

Cells were pre-incubated in sucrose solutions or salt solutions before chilling at a constant cooling rate to 0°C. A dip in thermal shock resistance was observed after 5 minutes in the salt solution. Thermal shock sensitivity increased with incubation in sucrose. Morris and Farrant (1973) concluded that results from cooling were attributed to pre-incubation. Glycerol and DMSO were unable to impede thermal shock damage Lovelock (1953); Farrant (1972).

A dip in survival with glycerol incubation was reported in human and bovine erythrocytes with respect to freeze/thaw survival Mazur (1974); Miller (1976). This dip was not observed when freezing was conducted in sucrose Mazur (1974). There were differences between the nature of these two dips.

First the time at which the dip appeared during incubation was peculiar to the treatment. Human erythrocytes demonstrated an immediate sensitivity to freeze/thaw, while thermal shock sensitivity dip was delayed until five minutes had elapsed Morris (1973); Mazur (1974).

Second, the amplitude of this dip in freeze/thaw and thermal shock sensitivity could be adjusted by increasing the concentration of either the cryoprotectant, glycerol or salt. However, with bovine erythrocytes not only did glycerol attenuate the dip but it delayed its appearance Mazur (1974).

In thermal shock this dip in salt solution incubation was unique in its admission of macromolecules. 'It may be possible that the Mazur/Leibo freeze/thaw dip was also the result of a weakened membrane. This could be tested by conducting the freeze/thaw experiments with trypan blue. The problem with this hypothesis was the presence of leaks in the sucrose control though admittedly the concentration of sucrose (40%) was very high. Sucrose protected against thermal shock and freezing and thawing.'

Although freeze/thaw was conducted in sucrose solutions

where survival was independent of cooling velocity Morris (1970), Daw (1973) found that infiltration of labelled sucrose, electrolyte exchange and increase in mass of intracellular water were curtailed where survival was optimized. Meryman and Hornblower (1972) questioned the relevance of leaks. Where loss of 2,3 - diphosphoglyceric acid and electrolyte depletion were most pronounced in erythrocytes frozen in 14% hydroxethyl starch and 10% PVP, survival was also highest. One would also expect that trypan blue would be quite precise in predicting cell viability. Data supported the opposite supposition Calcott (1975); Harris (1974); V. Bohmer (1973).

If thermal shock accounted for toxicity encountered during slow cooling, chemicals that aggravate thermal shock should accentuate freeze/thaw damage.

Lovelock (1955) observed that lecithinase and cholesterol supplementation encouraged thermal shock in erythrocytes while lecithin addition reduced it. Chilling ram spermatozoa at 5°C incurred morphological alterations; acrosomal swelling, mitochondrial and plasma membrane disintegration. Addition of egg yolk, which contained lecithin, ameliorated this damage Jones (1973). Post-thaw shock experienced by frozen/thawed bovine spermatozoa could also be averted by egg yolk addition Senger (1976). Resistence to cold shock in boar spermatozoa was attributed to phosphatidylserine, but not phosphatidylcholine Butler (1975). In erythrocytes egg yolk and its constituents, phosphatidylserine and lecithin,

reduced thermal shock damage Morris (1975). In both boar spermatozoa and erythrocytes this protection was increased as concentration increased.

Darin-Bennet (1977) demonstrated an ability in boar spermatozoa to surmount thermal shock and freeze/thaw as membrane cholesterol was increased.

'If thermal shock were to account for the cellular damage observed during slow cooling, then phosphatidylserine should protect at slow cooling rates and increasing plasma membrane cholesterol should increase freeze/thaw resistence.'

Cold Shock Model

Meryman (1971) first suggested the development of leaks in response to membrane stress encountered during shrinkage. These leaks would admit non-penetrating agents which would then protect colligatively. Farrant (1973) proved that while leaks occurred in both sucrose and salt solutions, the impact of electrolyte exchange was mitigated in sucrose. He also reported a positive correlation between infiltration of sucrose and thermal shock or freeze/thaw hemolysis Daw (1973).

Farrant and Morris (1973) postulated that events occuring during thermal shock were synonymous with those found during slow cooling. Freezing subjected the cells to a series of continuously increasing hypertonic solutions. Survival was presumed to be a function of exposure time to high osmotic

solutions. Suspensions frozen quickly avoided the toxic effects of leaks. Cryoprotective agents diluted extracellular solutes, minimizing the crisis represented by infiltration.

Slow cooling should show no increased damage with subsequent slow thawing since the cell had already equilibrated. Dilution shock, however, would not be avoided due to macromolecule invasion. With rapid cooling, the equivalent to thermal shock would be manifested during thawing. During a slow thaw cells would be subject to the same membrane stress encountered during slow cooling and would form membrane leaks as a consequence. This could be avoided with a rapid cooling rate. Such treatment would curtail subsequent dilution shock Farrant and Morris (1973).

There are several grounds for disputing the credibility of this model.

Farrant (1973) assumed that the traditional quick cool/rapid thaw formula rendered best survival. The problem was that cells cooled rapidly could tolerate a greater range of thawing velocities than cells cooled at more moderate rates as in the case of human erythrocytes, yeast and Neurospora crassa Miller (1976); Mazur (1968); Leef (1978).

DMSO, unable to impede thermal shock effects, was a very good cryoprotectant at slow cooling rates Farrant (1972). Recent data would suggest, however, that it also

might act by diluting electrolyte invasion Mironescu (1977).

Resistence to freeze/thaw at slow cooling rates was increased as sucrose concentrations increased. Increasing the sucrose concentration from 35% to 45% in thermal shock experiments was correlated with increased sensitivity Morris (1972;1973).

"Minimum cell volume" was discounted as a plausible explanation for freeze/thaw lethality since there was a lack of correlation between the onset of thermal shock sensitivity and cell mass. Sucrose and salt solutions induced similar degrees of dehydration during hypertonic shock experiments. Thermal shock hemolysis in hypertonic salt solutions was consistent with changes in cell mass. Thermal shock recovery in a sucrose solution yielding 80% recovery had a cell mass which exceeded that of cells in hypertonic salt solutions resulting in 25% recovery Daw (1973).

Lecithinase and cholesterol supplementation aggravated thermal shock sensitivity while lecithin addition decreased it Lovelock (1955). While membrane damage might be accelerated, cell volume may not have been altered.

It would seem that while the leak hypothesis would explain damage inflicted during slow cooling, there were several unexplained anomolies. The most notable of these was the formation of solute leaks in the sucrose control, conspicuously absent in the hypertonic salt control. The other contradiction was the discrepancy between the trypan

blue dye exclusion assay and others in predicting freeze/thaw viability. Woolgar (1974) suggested that leaks might constitute irreparable damage.

II E Dilution Shock

Woolgar (1974) discovered that freeze/thaw hemolysis was reduced when frozen/thawed cells were resuspended in hypotonic salt solutions. Hypertonic sucrose solutions in saline were more protective than hypotonic salt solutions. When freezing and thawing was conducted in PVP, sucrose, or salt solutions of equivalent osmolarity, resuspension in isotonic salt solutions resulted in similar survival. This would suggest that the cryoprotective properties of PVP and sucrose were instrumental in retarding dilution shock and related to the extracellular electrolyte concentration. Dilution shock resistence was closely correlated with the osmolarity of the resuspending solution. A corollary with hypertonic shock was established when it was found that cells subjected to hypertonic salt solutions resulted in higher recovery when resuspended in hypertonic solutions of PVP or sucrose. Resuspending in hypertonic salt solutions resulted in greater damage Woolgar (1974).

Woolgar (1974) suggested that thermal shock membrane leaks were symptomatic of a slow cool. Admission of large macromolecules predisposed the cells to dilution or colloid osmotic shock hemolysis upon thawing, averted only be resuspending in hypertonic solutions at room temperature.

He postulated that the membrane was automatically resealed and the degree of water and electrolyte exchange were counteracted by resuspending in hypertonic non-electrolyte solutions.

Resuspending in a hypertonic salt solution while retarding the intensity of membrane stress from water permeation, would not protect the cell from intracellular hypotonic ion concentrations. Resuspending in a hypertonic salt solution would protect the cell against these high intracellular electrolyte concentrations but would not reduce water permeation. Hypertonic solutions of sucrose in salt would not only protect against hypertonic intracellular electrolyte concentrations but would regulate the degree of water permeation. Dilution shock was important when cooling the following cells: human erythrocytes in sucrose; mouse lymphocytes in DMSO, human erythrocytes in PVP and dextran, rabbit embryos in DMSO, fetal rat pancreases in 2 M DMSO, bovine spermatozoa in glycerol, platelets in 6 DMSO and rat embryos in DMSO Miller (1976); Thorpe (1976); Pribor (1974;1973); Bank (1974); Mazur (1976); Bower Jr. (1973); Crowley (1975); Whittingham (1975). With the exception of erythrocytes all cells had an optimum cooling rate of less than 10°C/minute (Table 1).

ATPase acitivity on human red cell membranes varied with cooling rate as documented by Takehara (1971). If colloid osmotic shock accounted for cryoinjury, then membrane

ATPase activity of freeze/thaw and osmotic shock erythrocyte ghosts should be similar. Pribor (1971) tested this prediction and found a difference in enzyme activity between the two treatments.

If Woolgar (1974) were correct in postulating dilution shock at slow cooling rates, then at rapid cool/rapid thaw rates there should be less post-hypertonic shock. Dilution shock should be reduced. Pribor (1973) demonstrated that human erythrocytes were more susceptible to this kind of shock in 5% and 10% solutions of dextran and PVP at slow cooling rates. At higher concentration of 15% and 20% the opposite was found Pribor (1973).

Woolgar (1974) modified his model suggesting PVP and sucrose acted extracellularly. Solute infiltration was detrimental to recovery. PVP and sucrose at slow cooling rates would increase the number of membrane leaks. To avoid this, optimum cooling velocities were shifted to faster rates. The cryoprotective properties of polymers at rapid cooling velocities were attributed to a retardation in ice crystal growth. This was contradicted by the very high recovery observed at slow cooling rates in high concentration of polymers Morris (1972).

III THE EFFECT OF COOLING AND THAWING RATES, CRYOPROTECTANT AND CELL TYPE ON FREEZE/THAW SURVIVAL

The purpose of this part of the introduction is to review

the data used to support the physical chemical models previously discussed.

Three variables, thawing and cooling rate, cryoprotectant and cell type influenced cell freeze/thaw survival. Thawing rate will be discussed as a function of cooling rate.

III A Cooling Rate and Cell Type

I compiled a survey of optimum cooling rates for cells. Table 1 demonstrated that each cooling velocity/survival curve had at least one clear cell specific optimum. This proved beneficial when separating cell populations into B or T lymphocytes in 10% DMSO by cooling. Concavalin A (Con A) stimulated T cells into proliferating while Pokeweed mitogen (PWM) stimulated both. While the PWM mitogen response persisted after cooling at 4.4°C/minute, the Con A response disappeared, inferring the selection via freeze/thaw of B lymphocytes Knight (1972). This separation was further amplified with ammonium chloride pre-treatment Strong (1974).

The correlation between two-step cooling and the cooling velocity curve was verified when it was discovered that either optimum holding time or holding temperature were cell specific. While Chinese hamster cells and peripheral lymphocytes frozen in 5% DMSO had an identical holding temperature of -26°C, the holding time was 2 and 10 minutes, and 10 and 30 minutes respectively Farrant (1974); McGann (1976). Chicken skin cells, however, had a different optimum

holding temperature of -5°C Taylor (1960).

The "two factor hypothesis" was contingent upon the observation of one optimum cooling velocity. The demonstration of a second more subtle optimum, one which was independent of thawing rate and obscured by high concentrations of DMSO, might dispute this model's credibility. This was observed in bacteria, yeast, mycoplasma, human and bovine erythrocytes, Chinese hamster lung fibroblasts, human fibroblasts, and human lymphocytes Calcott (1974;1976); Mazur (1968); Raccah (1975); Miller and Mazur (1976); Mazur (1970;1974); Mazur (1972); v.Bodmer (1974); Knight (1972). The persistance of this peculiarity in the absence of DMSO attested to its cellular origin. Table 1.

Not only was freeze/thaw survival peculiar to cell type, it was also a function of cell cycle. Whereas Chinese hamster lung cells frozen in 15% glycerol at 5°C/minute were more sensitive in G2, HeLa cells frozen slowly in 10% DMSO were more susceptible in G1. Both were quite resistent to freeze/thaw during the S phase when DNA synthesis occurred Kock (1970); Terasima (1977). Since the collage of membrane antigens varied with cell cycle in rat basopholic leukemia cells as reported by Iserkey (1975), this 'might implicate glycoproteins in freeze/thaw survival.'

Survival was an indigenous cell quality (Mazur 1970) one which could be enhanced by altering the thawing rate, adding cryoprotectant, or by pre-treating with mitogens when

appropriate. Where a second optimum was observed, survival was unaltered when thawing rate was varied. High concentrations of cryoprotectant obscured this second optimum Knight (1972); Mazur (1972); Miller and Mazur (1976).

III B Rapid Cooling Rates and Thawing Rates

To avoid recrystallization of ice crystals, Mazur (1970; 1972) predicted that quickly frozen cells must be thawed rapidly. Corroboration was found in yeast, Neurospora crassa, human erythrocytes in 2 M glycerol, Chinese hamster lung fibroblasts frozen in various cryoprotectants, bovine spermatozoa, and bone marrow cells Mazur (1968); Miller (1975); Mazur (1972); Robbins (1976); Leibo (1970) and Leef (1978). Cell death and the probability of intracellular ice formation were correlated at faster than optimum cooling rates in several cells Mazur (1977).

Studies of cell ultrastructure after rapid cooling also substantiated this model. When cells were frozen quickly, freeze-etching and substitution confirmed a propensity for intracellular ice formation. Slow freezing favored cell shrinkage. Studies were conducted in Ascaridii gallii eggs in 5 and 10% DMSO, bull spermatozoa in glycerol, human erythrocytes in glycerol, Chinese hamster lung fibroblasts, yeast, and mouse ova Cruthers (1974); van Dujn Jr. (1974); Nei (1976); Bank (1972;1973). Two-step cooling showed similar behavior in Chinese hamster lung fibroblasts and mouse lymphocytes Farrant (1977); Walters (1975).

The temperature where recrystallization became harmful and susceptible to slow thawing was -40°C. Only above this subzero temperature did slow warming become deleterious to Chinese hamster cells, yeast, and Neurospora crassa survival Mazur (1972; MacKenzie (1970); Barnhardt (1971).

Two-Step Cooling

Chinese hamster lung fibroblasts were held at intermediate subzero temperatures of -25°C or -35°C for ten minutes before plunging into liquid nitrogen. These cells were thawed at various rates. Only the cells preincubated at -25°C exhibited a pronounced sensitivity to slow thawing (1% recovery) compared to 78% upon rapid thawing. The -35°C bath yielded 70% survival regardless of thaw. Freeze substitution micrographs demonstrated severe cell shrinkage and intracellular ice formation in both treatments Farrant (1977).

When Chinese hamster lung fibroblasts held at -25°C or -35°C, were subjected to various second holding temperatures prior to freeze/thaw, sensitivity to slow thawing was determined by the last subzero incubation temperature. This would suggest that cryoinjury was not due to recrystallization at pre-freezing treatments. Those cells held at -25°C followed by an incubation at -35°C, did not retain a sensitivity to slow thawing. If the second holding temperature succeeded the first freeze/thaw, all treatments were sensitive to slow thawing provided the second temperature exceeded -25°C Farrant (1977).

'One might speculate that the degree of dehydration might be conferred by the initial holding temperature. Upon slow thawing the cells would be prone to recrystallization because freeze-etching shows the formation of intracellular ice Farrant (1977). The intensity of dehydration would be curtailed at lower temperature baths prior to freezing. Another alternative may be that cells incubated at -25°C became so dehydrated, they succumbed to solution effects upon thawing.'

One contradiction to the rapid cool/rapid thaw model was that at very high cooling velocities, far exceeding that predicted for intracellular ice formation, survival with slow thawing was slightly higher than that observed at moderate cooling rates. This was seen in Saccharomyces cerevisiae at a cooling rate of 1000°C/minute, Neurospora conidia at 500°C/minute, and human erythrocytes in 1 to 2 M glycerol at 10^4°C/minute Mazur (1968); Tokio Nei (1976); Leef (1978).

Mazur (1977) attributed cryoinjury at rapid cooling rates to mechanical disruption from intracellular ice crystals formation. Farrant (1973) suggested that dilution shock from melting crystals accounted for the damage. Since neither explanation adequately accounted for the above anamoly, Tokio Nei (1975) and Shimada (1975) suggested that the size of the crystal was an important factor. It has been lately proposed that ice formation did not in itself

Data from mouse embryos, Chinese hamster lung fibroblasts in PVP and sucrose, red blood cells in 2 M glycerol, yeast and mouse lymphocytes demonstrated that recovery was dependent on the thawing rate used Bank (1974); Mazur (1972); Miller (1976); Mazur (1968); Thorpe (1976).

Miller (1976) found that at intermediate cooling rates of 480°C/minute and 180°C/minute survival appeared independent of the thawing rate used. Optimum cooling velocity was about 500°C/minute when a rapid thawing rate was used.

Rapatz and Luyet (1975) demonstrated a similar sensitivity to fast thawing with human erythrocytes in 1 M glycerol equilibrated at room temperature before cooling and thawing at various rates to different low temperatures. Above -40°C, slow cool/slow thaw always resulted in the best survival. Rapid cool/slow thaw or rapid cool/rapid thaw yielded low survival.

'In my opinion sensitivity to 100°C/minute thawing rate after slow cooling is not explained by the "two factor hypothesis".' The cooling rates were far slower than those predicted for intracellular ice formation Mazur (1977). If this indeed were the case, then at slightly faster cooling rates survival should not be insensitive to thawing; as was reported by Miller (1976).

Rapatz and Luyet (1975) explained their data by assuming a nucleation temperature of -40°C. Fast cool/fast thaw above this temperature exposed the cell to huge

mechanical stress resulting in membrane rupture and solute influx. Colloid osmotic shock would be avoided using faster cooling rates, slower thawing rates or cryoprotectant infiltration into the cell.

Both of these experiments were characterized by room temperature incubation in glycerol for at least one half hour Miller (1976); Rapatz (1975). Incubating cells at room temperature allowed glycerol to permeate into the cell Lovelock (1955); Mazur (1975). If these results represent an artifact of penetration which would mitigate colloid shock, then cells incubated at 0°C should be more sensitive to fast thawing. If the intracellular glycerol contributed to colloid shock, then the difference between rapid thaw and slow thaw survival should be less when no intracellular was present. Cells without glycerol should be less sensitive to fast thawing. The Mazur (1972) data with Chinese hamster lung fibroblasts incubated at 0°C before cooling supported the former supposition. Sensitivity to fast thawing was evident in the absence of infiltration. The Wood (1978) data suggested that slow cooling to -80°C might expose the cells to greater dehydration and upon thawing, colloid osmotic shock. Dilution shock would be decreased with slow thawing. This does not 'in my opinion' explain resistance to slow cooling at thawing rates of 100°C/minute in human erythrocytes Miller (1976).

III D Cryoprotection

Rapatz and Luyet (1965) divided cryoprotective agents into two groups; those that penetrate the cell, protecting at slow cooling rates; and those, that are non-penetrating, protecting at higher cooling velocities. Glycerol and DMSO describe the former group, while sucrose and dextran characterize the latter one. This model was deduced from survival of erythrocytes cooled in various cryoprotectants, and survival of CHO cells, human erythrocytes, and Mycoplamsa incubated at high subzero temperatures. Only when penetration of glycerol had occurred did these cells survive incubation for any period of time at -30°C Rapatz (1965); Lovelock (1953); Taylor (1974); Raccah (1975).

The relevance of these data to cooling and thawing is questionable since penetration was not always correlated with survival at slow cooling rates as demonstrated by bovine erythrocytes, bovine spermatozoa, CHO cells, human erythrocytes, and mouse embryos Mazur (1975;1974); Wiggin (1975); Senger (1976); Mazur (1972); Leibo (1974).

When human erythrocytes were incubated at 0°C in 1.4 M sucrose or 2 M glycerol for various times preceding cooling at 60°C/minute, survival was not correlated closely with amount of intracellular glycerol Miller (1976); Dalgliesh (1972). Freeze/thaw sensitivity after glycerol incubation was greatest immediately following addition. Survival decreased as pre-cooling incubation time in sucrose increased.

Mouse lymphocytes incubated in various amounts of DMSO at 0°C before freeze/thaw showed a direct relationship between survival and DMSO concentration up to 5%. After five minutes, survival was independent of DMSO concentration Thorpe (1976). With human renal epithelial carcinoma cells, sensitivity to slow thawing was proportional to DMSO concentration Rozner (1978).

The credibility of cryoprotectant permeability was also disputed by freeze/thaw survival of human erythrocytes in higher concentrations (20%-40%) of non-penetrating cryoprotectants. At these concentrations the optimum cooling rate was shifted to slower cooling velocities Pribor (1974); Morris (1970).

There are several ways in which cryoprotectants consistently alter freeze/thaw survival. As the concentration of cryoprotectant was increased, the optimum cooling rate was much slower, reducing survival normally observed at higher cooling velocities. This observed in human lymphocytes, Chinese hamster cells, bovine spermatozoa, bone marrow cells, mouse lymphocytes, and human erythrocytes Knight (1972); Leibo (1970); Rodriguez (1975); Leibo (1970); Thorpe (1976); Pribor (1974;1976). If double optima were observed, higher concentrations of DMSO or glycerol tended to obscure the optimum observed at slow rates Knight (1972); Mazur (1972); Miller (1976). Very high concentrations of all observed cryoprotectants favored 100% survival regardless of cooling

rate in erythrocytes Pribor (1976); Tokio Nei (1975); P. Mazur (1974); Morris (1970).

If the cooling velocity were constant and the cryoprotectant concentration were increased, survival accordingly increased and reached a plateau. At even higher concentrations, toxic effects were observed. This was seen with mouse and rabbit embryos, bovine spermatozoa, fetal rat pancreases, human erythrocytes, mouse lymphocytes, bovine erythrocytes, and Chinese hamster ovary cells Bank and Maurer (1973); Robbins (1976); Mazur (1976); Pribor (1976); Morris (1970); Hein (1976); Odink and Sprokhclt (1977); Ashwood-Smith (1972); Connor (1973).

Colligative effects such as osmolarity or hypertonicity did not fully explain the protection attained by cells Pribor (1976); Miller and Mazur (1976); Farrant (1972); Meryman (1971). Meryman (1974) reported that less than colligative concentrations of protectant achieved considerable protection. Colligative behavior with DMSO was predicted only at concentrations exceeding 2 M (15%) Cocks (1974. Yet much smaller concentrations such as 10% DMSO yield considerable survival Harris (1977); Mazur (1972).

Protection has also been attributed to hydrogen binding which would increase the volume of liquid phase, to kind of ice crystal formation, to limiting ice crystal growth due to viscosity or to osmotic shock protection Meryman (1971); Farrant (1973); Mazur (1977); Mironscu (1977;1978).

Glycerol and DMSO induced startling membrane changes such as particle clustering on mouse lymphocyte plasma membranes. Removal of cryoprotectant reversed the effect McIntyre (1974). Takehara (1975) reported clustering of methyl orange receptors following freeze/thaw of erythrocytes. The number of binding sites did not decrease. In Tetrahymena pyriformis unsaturated fatty acid incorporation was correlated with successful acclimation and decreased particle clustering compared to untreated control Kitajuma and Thompson (1977). 'Particle clustering may be a result of the cryoprotectant and unrelated to freeze/thaw survival.'

Protection by Fetal Calf Serum

Human lymphocytes, mouse lymphocytes, Chinese hamster cells, and bone marrow cells were protected from freeze/thaw injury by fetal calf serum Glikerson (1977); Thorpe (1976); Ashwood-Smith (1972); Damjonovic (1974).

As mouse lymphocytes suspended in 10% DMSO plus various concentrations of fetal calf serum were cooled at faster rates, survival decreased. This may be an attribute of DMSO protection. Addition of fetal calf serum from 5% to 25% yielded the expected increase and plateau Thorpe (1976).

Decreasing the concentration served to shift the optimum cooling rate to a slower one by 'implication'. At a cooling rate of 5.8°C/minute, survival was 35.1% and 33.2% in 5 and 10% serum respectively. At 1.2°C/minute survival was 56.1%

and 63.4% in 5 and 10% DMSO respectively.

Serum (10%) had a slight protective effect against dilution shock. At 0°C, only concentrations exceeding 25% were effective in counteracting dilution effects Thorpe (1976). The cryoprotective ability of serum cannot be attributed wholly to deferring colloid osmotic swelling.

Glikerson (1977) performed similar experiments with bone marrow cells at only one cooling rate. The control yielded 33.3% recovery compared to 48.8% and 53% survival in 15% and 50% serum respectively.

Serum also provided some protection in two-step cooling although the optimum conditions were at -10°C for 2 minutes Knight (1977).

The counterpart to serum in freezing might be egg yolk which protected against cold shock during chilling. Both glycerol or buffer acted synergistically with egg yolk as DMSO did with fetal calf serum. Morris (1975) established that phosphatidylserine protected erythrocytes in much the same manner as did egg yolk M.M. Pace (1974). Confirmation of this relationship might provide some tangible link between cold shock and freeze/thaw.

IV BIOCHEMISTRY AND ULTRASTRUCTURE OF CELLS WITH FREEZE/THAW

The purpose of this section is to establish a relationship between optimum cooling rate and cell damage.

Ultrastructure and biochemistry have been used to elucidate this problem. One could formulate two approaches to this question. One postulate would favor a cell specific site of freeze/thaw damage, readily manipulated biochemically and peculiar to cooling rate. The other alternative would be to attribute cell death to cumulative nonspecific cell damage.

IV A Ultrastructure

Ultrastructure of frozen/thawed cells has been examined and correlated with cell survival at different rates. The limitation of the technique, however, resided in the small sample size and the inability to ascertain cell viability directly.

Increased cell size following slow freeze/fast thaw was observed in polymorphonuclear granulocytes and HeLa cells following quick freeze/thaw Loinetti (1975); Shimada (1972). Membrane blebbing also was observed in HeLa cells and Chinese hamster cells in 10% DMSO following freeze/thaw Hunt (1977); Shimada (1978).

Microtubule destruction, nuclear disintegration, mitochondrial and endoplasmic reticulum dilation, membrane disruption characterized frozen/thawed Chinese hamster ovary cells in 10% DMSO frozen at 1.7°C/minute, mouse soleus muscle, ram semen frozen slowly, fowl spermatozoa frozen quickly, pancreatic rough endoplasmic reticulum frozen quickly and platelets Hunt (1977); H. Bank (1972); W.D. Bowers (1973);

Nath (1972); Harris (1973); Sherman (1973); Crowley (1974). Dog granulocytes and Chinese hamster ovary cells following slow freeze/rapid thaw demonstrated accumulation of granule and cytoplasmic lipid inclusions French (1973); Crowley (1974).

A lethal target could be determined by identifying cell organelle damage conspicuously absent in treatments yielding high survival. Damage to the endoplasmic reticulum was observed in such samples Bank (1973). Hunt (1977) also observed condensed but not dilated mitochondria. Lipid inclusions, mitochondria dilation, membrane disruption were absent when survival was high. Similarly mitochondrial disintegration and membrane blebbing present in unprotected frozen/thawed ram and fowl spermatozoa disappeared with the inclusion of egg yolk and DMSO in the suspension regime Jones (1973); Harris Jr. (1973).

Some of these ultrastructural changes might be attributed to the cryoprotectant. In Rhesus kidney cells and mouse lymphocytes, DMSO promoted membrane blebbing Malinin (1973); MacIntyre (1974); Crowley (1974). Membrane blebbing in CHO cells was correlated with high freeze/thaw viability. It is unlikely that membrane blebbing constituted irreparable damage.

In concentrations of DMSO exceeding 15%, dilated mitochondria and endoplasmic reticulum were observed in Rhesus kidney cells. DMSO also stimulated autophagocytic activity at

25°C and lysosome accumulation at 4°C Malinin (1973). Lysozyme induction in 1% DMSO was seen in some mouse myeloid leukemic cells Krystosek (1976). 'Since it is unlikely that a cryoprotectant would initiate changes adverse to freeze/thaw survival, the probable target was the plasma membrane.'

IV B Biochemistry

Chinese hamster ovary cells demonstrated dense staining of the heterochromatin following slow freeze/thaw implicating mRNA synthesis and ultimately protein synthesis Hunt (1977). That protein damage occurred was indicated by Harris (1974; 1977). Chromium ion release, implying protein denaturation, correlated with results from other freeze/thaw viability assays. It is important to distinguish between protein denaturation and protein leaks.

There are three arguments against this proposal in my opinion. One was the presence of degranulated endoplasmic reticulum in treatments rendering high survival Bank (1972). Another was the indifference of repair in CHO cells to oxygen concentrations between 0.6 uM and 277 uM (aerobic) although repair was maximized at 37°C expected if enzymatic operations were mandatory McGann (1975). Finally DMSO stimulated mRNA synthesis in bacteria and Friend virus transformed cells implying a cryoprotectant artifact Travers (1974); Keppel (1977).

In slowly frozen bacteria, Toshkiki (1973) found inhibitors of RNA and protein synthesis impaired Streptococcus in rat bone marrow cells with acid phosphatase activity, and in rat macrophages with membrane receptors after freezing at 2°C/minute Robbins (1976); Harris (1974); Fishbein (1976); Kahn (1976); Tadashi Araki (1977); Nath (1972); Persidsky (1971); Hein (1977).

'One might expect that if membrane integrity were of major importance, trypan blue should give an accurate appraisal of survival.' This was demonstrated to be untrue in rat macrophages, human diploid cells, CHO cells, and MRC cells Hein (1977); von Bohmer (1973); Harris (1974). Protein elution was not evident in erythrocytes after freeze/thaw Meryman and Hornblower (1972). 'If membrane continuity were compromiseddurin freeze/thaw, it would have been better to include trypan blue in the actual procedure, eliminating deviance due to immediate resealing of the membrane upon thawing.'

Inclusion of phosphatidyl inositol or sialic acid failed to influence postthaw recovery Robinson (1973). This data might be queried since phosphatidylcholine and phosphatidyl-ethanolamine were the major constituents of many membranes studied Bruckdorfer (1976); Vance (1977); Rothman (1977).

In Saccharomyces cerevisiae, ram spermatozoa, rat liver mitochondria, a preponderance of lysophosphatides were observed after quick freeze/thaw. Bull spermatozoa, resistent

to thermal shock displayed a concomitantly low amount of lysophophatides Hiroshi Souzu (1973); Milovanor (1975); Araki (1977). Morris (1978) reported large amounts of free fatty acids in Chorella following slow freeze/thaw. Free fatty acids have been reported to cause death in tissue culture and lysophosphatides have been noted to interfere with cell adhesion, and therefore, cell cloning. Moskowitz (1967); Curtis (1975). Yoshida (1974) found that membrane phospholipases were activated by freeze/thaw in plants.

A change in the membrane itself was not necessarily detrimental to cell survival as demonstrated by rat macrophages and human red cells Hein (1977); Takehara and Rowe (1971). ATPase activity on human red cell membranes varied with freeze/thaw. The greatest deviation from normal cell activity was experienced when the optimum cooling rate of 330°C to 440°C/minute was approached. It might appear that a membrane conformational change was fundamental to survival since cryoprotectant also depressed ATPase activity. This decrease could not be explained by protein elution upon freezing. In Neurospora crassa, filipin which binds to membrane sterol altered susceptibility to fast freeze/slow thaw damage Barnhardt (1971).

Since membrane disruption, as assayed by protein elution, trypan blue dye exclusion, and enzyme activity failed to simulate cell survival accurately, 'toxicity may be insidious from post-thaw lipid waste products.'

IV C Mitochondria

Fishbein (1968;1969) demonstrated that succinate cytochrome c reductase complex, linked to oxidative phosphorylation in mouse liver mitochondria, could be used to assay freeze/thaw damage. The optimum cooling velocity was 100°C/minute. In 1971, Fishbein froze whole rat liver slices at 1°C/minute to -25°C in 0.25M sucrose and various amounts of DMSO. Recovery was maximized in 15% DMSO.

When mouse liver mitochondria were extracted and similar experiments were performed, much higher velocities were required to induce the same damage (1300°C/minute to 5000°C/minute). Although sucrose was able to mitigate ultrastructural damage, the loss of succinate cytochrome c reductase activity and oxidative phosphorylation was unabated Fishbein (1976).

Tadashi Araki (1977) froze rabbit mitochondria, assaying respiration rate using two cytochrome electron transfer complexes: succinate cytochrome c reductase and NADH cytochrome c reductase with ketoglutarate as substrate. Both displayed a similar sensitivity to freeze/thaw. When individual enzymes of these complexes were examined in frozen/thawed mitochondria, the diminished ketoglutarate dehydrogenase activity could be explained by elution, while succinate hydrogenase activity loss was due to conformational membrane changes altering substrate site accessibility.

That damage occurred to mitochondria during freeze/thaw is undisputed. Whether it constitutes irreparable damage is

dubious. If the mitochondria were the lethal target one would expect that isolated mitochondria would produce a similar freeze/thaw profile to intact cells. Both Fishbein (1976) and Araki (1977) demonstrated no similarity. Secondly, cells showing dilated mitochondria should not be viable upon thawing. Both surviving adult rat hepatocytes in 10% DMSO and fowl spermatozoa had dilated mitochondria Le Cam (1976); Harris (1973). Thirdly, survival should depend upon oxygen concentration McGann (1975). Finally, cryoprotectant should not induce dilation as reported in Rhesus kidney cells Malinin (1974).

IV D Lysosomes

M.D. Persidsky and M.E. Ellet (1971) proposed lysosomes as primary cause of cell death in rat bone marrow cells. Cells were frozen at 1°C/minute to -35°C, transferred to -80°C, and thawed quickly. Intracellular inclusion of trypan blue or acetyl salicylic acid decreased supernatent acid phosphatase after freeze/thaw. Morris (1978) correlated freeze/thaw survival in Chorella with the presence of a vacuole which he identified as a lysosome. Lysosomal damage was also conjectured to be the cause of death in polymorphonuclear granulocytes, platelets, and rat hepatocytes Crowley (1974); Dayran (1976); Le Cam (1976).

Unfortunately DMSO, in cryoprotective concentrations, activated lysosomes Malinin (1973); Krystosek (1976).

Subjecting rat liver lysosomes extracts to various freeze/thaw regimes did not yield the expected optimum cooling rate, although 7.5% DMSO alleviated damage at slow cooling rates Osborne (1973). High but cryoprotective concentrations of sucrose and glycerol disrupted platelet and rat liver lysosome membranes Dayran (1976); Osborne (1973).

A definitive test for this model in my opinion would be to compare freeze/thaw survival of the DMSO plus or minus myeloid leukemia clones. DMSO induces lysosome induction Kyrstosek (1976). Recent reports have implicated the existence of membrane receptors for insulin, epidermal growth factor and DMSO Maxfield (1978); Bari (1978); Sachs (1978).

IV E Endoplasmic Reticulum

Sherman (1973) froze rat pancreatic endoplasmic reticulum after suspending in glycerol at 4°C and room temperature. Intracellular glycerol, like DMSO, caused dilation and degranulation, more pronounced at faster cooling rates. The morphological toll was least in slow freeze/rapid thawing regime. Although least effective during slow freeze/rapid thaw, extracellular glycerol preserved structure throughout all regimes.

The importance of this damage should be implied by the production of lipids and secreted proteins Craig (1978); Bruckdorfer (1976). The number of membrane receptors was decreased in rat macrophage after slow freeze/thaw Hein

(1974). Insulin receptors in rat hepatocytes were dependent upon freezing regime Le Cam (1976). It is not clear rather the synthesis of receptors was inhibited.

A test for identifying lethal damage was to initiate a molecular change which would alter survival from freeze/thaw. In Neurospora crassa the Poky mutant, a leaky osmotic membrane mutant, has a varied suspectibility to freeze/thaw. This was observed in fatty acid mutants of Saccharomvces cerevisiae Kruuv (1978).

In bull spermatozoa, Darin-Bennet (1977) established a parallel between cholesterol content and thermal shock resistence. A high polyunsaturated/saturated fatty acid ratio accentuated cold shock sensitivity. The opposite relationship was demonstrated in mycoplasma with respect to cholesterol. At slow cooling rates, cholesterol sensitized mycoplasma to slow freeze/thaw. Survival was augmented with a high unsaturated fatty acid/saturated fatty acid ratio at rapid cooling rates Raccah (1975). Goldberg and Eschar (1977) with lactic bacteria, found the same to be true. Yeast grown anaerobically gave higher freeze/thaw survival with higher unsaturated fatty acid/saturated ratio in the membrane. Aerobically grown yeast demonstrated the opposite effect Kruuv (1977). In Chorella, survival was independent of fatty acid constitution Morris (1978).

In eukaryotes, addition of mitogens which attach to glycoproteins in the lymphocytes membrane, altered the optimum cooling rate Knight (1972). Addition of butylated

hydroxytoluene in Chinese lung fibroblasts resulted in a concomitant shift from 3°C/minute to 0.3°C/minute. When the fluidity of the membrane in Saccharomyces cerevisiae was similarly altered, survival changed but the cooling rate profile was maintained Kruuv (1978); Frim (1976).

MATERIALS AND METHODS

Chinese hamster ovary cells, Puck's Clone A, a gift from Dr. D.M. Robinson, Georgetown University, U.S.A., were used in the following experiments.

Cells were grown in 25 ml tissue culture flasks or dishes (Falcon) in medium referred to as CTM. CTM consisted of Eagle's Minimal Medium (Gibco) supplemented with kanamycin (Sigma Chemicals); 45 mM Tricine (Sigma Chemicals, a buffer; 1.8 mM glutamine (Sigma Chemicals); 0.08% sodium bicarbonate (Fisher Chemicals); and 10% fetal calf serum (Gibco).

Responses to various treatments were assayed using the following techniques: cell propagation; vital dye exclusion as measured by trypan blue; and cloning, formation of macroscopic colonies ten days after incubation. Each colony was assumed to represent the division of a single cell. Fundamental to all these techniques was a procedure called cell harvesting.

Cell Harvesting

After cells had grown to exponential or log phase in flasks containing 7 ml of CTM, the conditioned medium was removed with a pipette (Fisher Chemicals) under vacuum. Two ml of a 0.5% Bacto-trypsin (Difco) in salt solution without magnesium or calcium, were added and diluted with an equal volume of CTM when all the cells had detached from the flask. This suspension was then transferred using a Pasteur pipette (Fisher Chemicals) to a Pyrex screw top tube to be

centrifuged at 700g for three minutes in a bench type laboratory centrifuge. The pellet was resuspended in fresh medium after removing the supernatent under vacuum. These manipulations were conducted aseptically with the protection of a laminar flow hood.

Cell Propagation

After harvesting, cell count was determined using a Bright-line hemocytometer. An appropriate dilution was made and the required number of cells were dispensed into a number of tissue culture dishes or flasks. The cells were incubated for various times at 37°C in a carbon dioxide incubator. (National). A solution of 78% sodium bicarbonate/22% tartaric acid in distilled water was substituted for the CO_2 gas. At predetermined intervals cells were harvested and counted using the hemocytometer.

Trypan Blue Dye exclusion

A 0.4% solution of trypan blue in Hank's Balanced Salt solution was prepared. One tenth of 1 ml was added to 0.5 ml of a 2×10^5 cell/ml suspension. After five minutes, cell viability was scored by preforming a cell count to ascertain the percentage of blue cells, indicative of death.

Cloning

Cloning was the alternative method for assaying cell viability. A cell suspension of 10^4 cells/ml was prepared and 0.1 ml was added to a number of plates containing 5 ml of CTM. After a ten day incubation, the medium was decanted

and replaced with a 0.2% solution of methylene blue (Fisher Chemicals) in water. After at least 15 minutes, the supernatent was washed off with tap water, revealing stained colonies which were counted.

Lipid Depleted Medium

Lipid depletion was accomplished using two techniques.

A. Ammonium sulfate precipation. The technique followed was described by Williams (1975). After collecting the protein precipitate by centrifugation in a Sorvall centrifuge at least 3000 rpm, the pellet was resuspended in Hank's Balanced Salt solution and dialysed against this solution, followed by at least two batches of CTM without serum.

B. Ethanol: Ether Extraction

The ethanol ether extraction was performed as reported by Horowitz (1974). The protein obtained by this extraction was dried under vacuum and suspended in CTM minus serum.

Protein determinations were performed using the Lowry method (1951) and electrophoresis was conducted to ascertain whether or not protein selection had occurred Dietz (1967). Both sera were sterilized by millipore filtration (0.22 µm pore size) (Millipore). Medium was made to conserve protein concentration found in normal medium.

The following determinations were performed on sera after extraction with chloroform/methanol extraction.

1. Thin layer chromatograms of Silica gel (Eastman (Chromatogram) were developed using a solvent system of

petroleum ether; diethylether; acetic acid in 90:10:1.5 proportions. The chromatogram was visualized with iodine vapour.

2. Phospholipid determinations were conducted according to Ames (1960).
3. Fatty acid determinations were performed after extraction according to Dole (1956) and detected spectrophotometrically as described by Itaya (1977).
4. Cholesterol was determined by the digitonin method.
5. Electrophoresis was by the method of Dietz (1967). Gels were stained with Amido-Schwartz in 7% acetic acid and analyzed by a spectrodensitometer at 540 nm.

Fatty Acid Supplementation

Linoleic acid and palmitic acid (both from Fisher Chemicals) were added to the lipid free medium using the method of Spector (1969). Concentrations used were 20 nmoles of fatty acid/10 grams of Celite. After evaporating to dryness under N_2 gas or air, the Celite with attached fatty acids was incubated in CTM minus serum with published amount of fatty acid free bovine albumin. Extraction and sterilization of fatty acid albumin complex were achieved by millipore filtering.

A stock solution of avidin (Sigma Chemicals) in serum free CTM was prepared to final concentration of 10 to 12 international units/ml. The experimental concentration was 0.12 international units/ml of CTM.

Cell Determinations

Cholesterol determinations of treated CHO cells were performed in the following ways:

1. Cells were extracted with chloroform/methanol and spotted on a chromatogram. The chromatogram was developed in sulfuric acid vapour and read with a spectrodensitometer. The relative amounts of lipids were determined by integrating the areas under the graph.
2. Cholesterol determination were performed as previously described.
3. Relative cholesterol content was implied by susceptibility to cholesterol esterase on membrane fragments obtained through the courtesy of Sheila MacIntyre. (UVic Dept. of Biochemistry and Microbiology).

Fatty acid replacement on the cell membrane was inferred by employing the technique of Hoover (1977). Increased fatty acid unsaturation was thought to decrease cell/cell adhesion. The technique used in the experiment was modified in the following ways. Instead of the added cell suspension, the monolayers were treated to change membrane fatty acids by incubating in the experimental medium. The normal cell suspension was labelled with 0.15 ml of tritiated glutamic acid, 60 mCi specific activity, (New England Nuclear) in 3 ml of incubating medium. After leaving overnight to maximize entry, the cells were harvested in lipid free medium, and

resuspended in serum free CTM. A cell count was performed to determine label/cell. A known number of cells was added to the scintillation vials in which the control and experimental monolayers had been grown. After 20 minutes, the supernatent was removed and 1 ml of 1N NH_4CH was added followed by 10 ml of Unisolve. The radioactivity was counted in a scintillation counter.

Freeze/thaw Experiments

Cells were harvested and appropriate dilutions were made. A stock solution of 5% DMSO (Fisher Chemicals) in normal or experimental medium was prepared and sterilized by Millipore filtering. An equal volume of the DMSO solution was slowly added to the appropriate dilutions. Pre-cooling manipulations were performed at room temperature. The sample size allotted per provial (Western) for subsequent cooling was 1.2 ml. Approximately one thousand cells were pipetted to control plates for cloning. For each of the experiments performed, there was an unfrozen control.

Survival from freezing was defined as number of clones following freeze & thaw/number of control clones x 100.

A record of the freezing regime was collected by inserting a copper constantin thermocouple into a sample of CTM. The thermocouple was connected to a Honeywell recorder. Slow cooling rates of 1°C/minute and 5°C/minute were obtained by adjusting liquid nitrogen levels in a

Dewar Flask. Faster cooling rates of 13°C/minute and 25°C/minute were realized by using a CO_2/ethanol bath. The samples were seeded by mechanical agitation or by touching provials against the top of a metal container filled with liquid nitrogen. Cooling rate was defined as:

°C/minute from room temperature to -40°C.

RESULTS AND DISCUSSION

Ammonium Sulfate Treatment

The procedure followed was described by Williams (1975) with two differences. The ammonium sulfate precipitation was conducted only once for one half day and reconstituted to conserve the original protein concentration. Such treatment was purported to conserve protein activity as reported by Horowitz (1977), Temin (1972) and by electrophoresis.

Growing LM cells in 10% calf serum resulted in a 20% polyenoic fatty acid incorporation into the phospholipids. Supplementing with 15% ammonium sulfate (AMS) treated fetal calf serum reduced this to 4% or less. This was also true for 3T3 and SV3T3 cells. A three day incubation in medium with 15% AMS calf serum (AMS) increased the degree of fatty acid unsaturation in LM, BHK 21, 3T3, and SV 3T3 cells. All freeze/thaw experiments here were performed within 24 hours. Cell proliferation in AMS medium was severely retarded although increasing AMS serum concentrations aided in restoring normal cell division. Doubling time in AMS (10%) medium was twice that found in control (10% fetal calf serum). Supplementation with 8 µg/ml of Tween 19:0 retarded cell division (Williams 1975) as was demonstrated in Figure 2 with oleic acid (4×10^{-5}M) supplementation.

Horowitz (1977) suggested that this technique was not

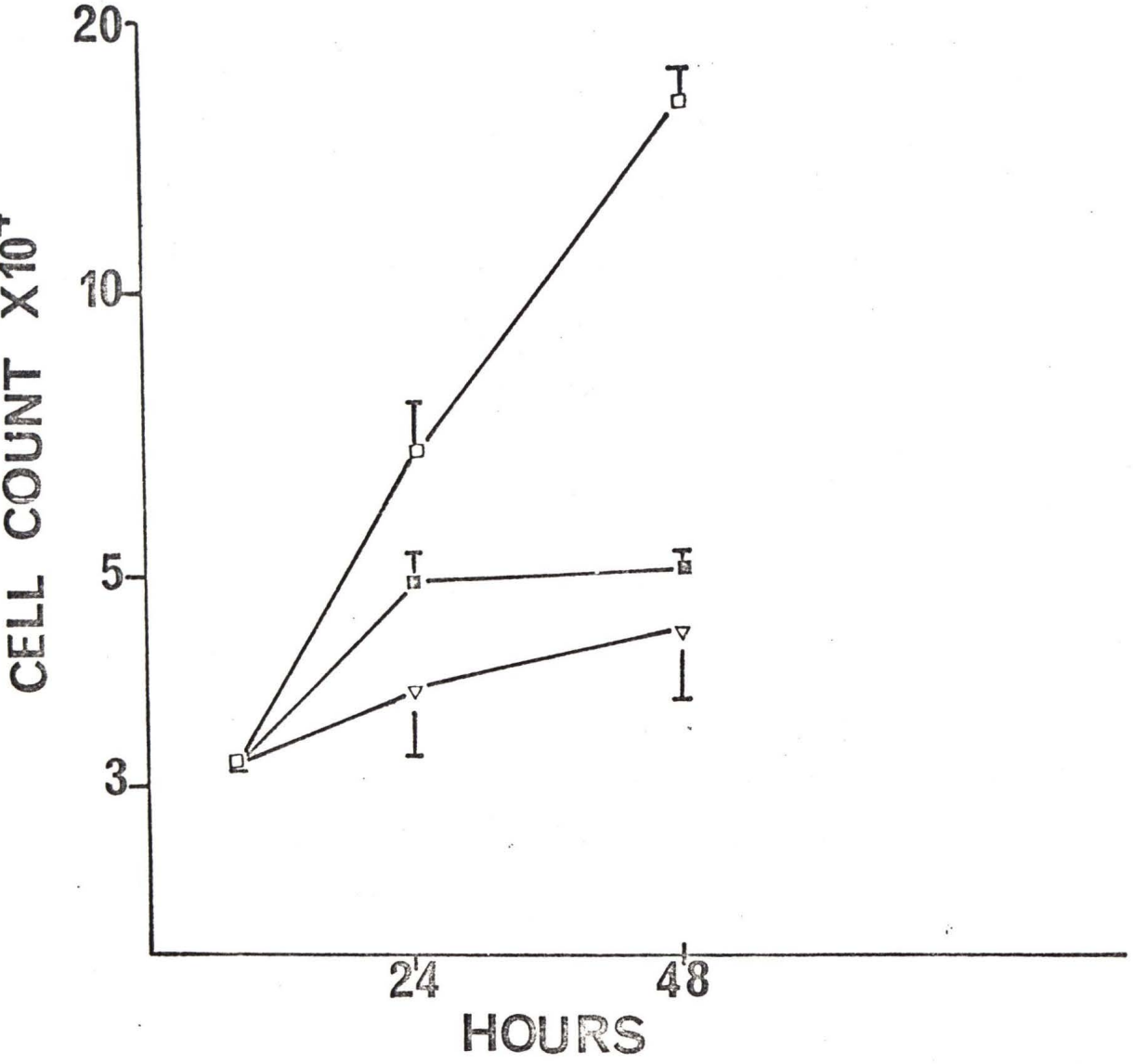
Figure 2. Cell growth in 4×10^{-5} M oleate in partially lipid depleted medium

□ Control

■ Partially lipid depleted medium

▽ Partially lipid depleted medium with oleate (4×10^{-5} M)

Error bars represent one standard deviation.


20
10
5
3
CELL COUNT X10^4
24
48
HOURS

reliable in reducing lipid content in serum. The biochemical tests performed confirmed this although phospholipid content was consistently diminished by 75%. Fatty acid depletion and cholesterol depletion seemed peculiar to each treatment although cholesterol/phospholipid ratio was consistently higher than in control. Appendix I.

The total cholesterol concentration in AMS serum was determined rather than unesterified cholesterol. Data from Bruckdorfer (1976), Rothblat (1972) and Martin (1977) with mitochondria, MB111 cells, L cells, L5 178Y and human fibroblasts suggested that cholesterol ester was hydrolyzed to free fatty acid and cholesterol which became incorporated into the membrane where sterol content would be regulated by fatty acyl CoA: cholesterol acyltransferase. Kirby and Green (1977) increased the erythrocyte membrane uptake by increasing the cholesterol/phospholipid while maintaining a constant lipoprotein content. Omitting DMSO deterred cholesterol exchange from the membrane to the medium but did not inhibit the reciprocal event from occurring Bruckdorfer (1976). The experiments performed here would imply that cholesterol content in cell membranes was increased. Appendix II.

Cholesterol had the added advantage of concentrating in the plasma and lysosome membrane of the cell with smaller amounts found in microsomes. The Golgi complex, endoplasmic reticulum, mitochondria, and nuclear membrane were characterized by miniscule amounts Jain (1975); Schroeder

TABLE II

CLONING EFFICIENCY IN VARIOUS MEDIA AFTER GROWING IN RESPECTIVE MEDIUM FOR 24 HOURS

Incubation Medium	Cloning Medium % Survival		
	Normal	Millipored	Ammonium Sulphate
Normal	45.4	56.6	0
S.D.	3.21	2.46	
Millipored	39.4	22.7	0
S.D.	7.40	0.63	
Ammonium Sulphate	65.9	34.7	0
S.D.	1.18	5.99	

TABLE 3

EFFECT OF ASM TREATMENT ON CELL CLONING AND VIABILITY

Time	Time of incubation in AS medium							
	0 hours		24 hours		48 hours		72 hours	
	C.E.	%V	C.E.	%V	C.E.	%V	C.E.	%V
0 hours	18.2	0.99						
S.D.	3.4							
24 hours	5.1	0.97	11.7	0.98				
S.D.	2.7		2.7					
48 hours	25.9	1.00	17.0	1.00	22.4	0.98		
S.D.	3.3		4.2		3.7			
74 hours	17.8	1.00	12.2	1.00	20.9	1.00	14.4	0.97
S.D.	2.0		3.3		1.9		7.4	
96 hours	41.2	1.00	40.0	0.99	40.9	0.97		
S.D.	2.8		1.1		2.2			

(1976); Thines-Sempoux (1973); Bruckdorfer (1976); Elias (1978). Most of the cholesterol was concentrated in the plasma membrane rather than the lysosome Henning (1970); Thines-Sempoux (1973); Schroeder (1976).

This system should, therefore, not only elucidate the role of cholesterol in freeze/thaw but implicate the site of freeze/thaw damage.

The following experiments were performed to investigate the effect of AMS medium upon CHO cells. Table 2 indicated that cells grown in normal, millipored, and AMS medium were unable to clone in AMS medium. That cells grown in AMS medium for 24 hours were able to clone in normal medium without a decrease in cloning efficiency implied that any cell changes were reversible with respect to adhesion and cell division.

The second parameter studied was cell proliferation Figure 3. Cells grown in AMS medium experienced a retardation in proliferation which disappeared after 48 hours. Immersing the cells in normal medium after 24 or 48 hours of AMS incubation demonstrated as immediate reinstatement of normal cell doubling time. Reference to Table III suggested that cell viability and cloning efficiency were not impaired during this incubation. These three tests suggested that although cell proliferation was diminished such an effect was not toxic and reversed after 48 hours.

Other delipidated medium has been reported to

Figure 3. Cell growth in partially lipid depleted medium replenished with normal medium.

□ Control

▼ Cells incubated for 24 hours in depleted

▽ medium

■ Cells incubated in depleted medium for 48 hours

Cells incubated in partially depleted medium

Error bars represent one standard deviation.

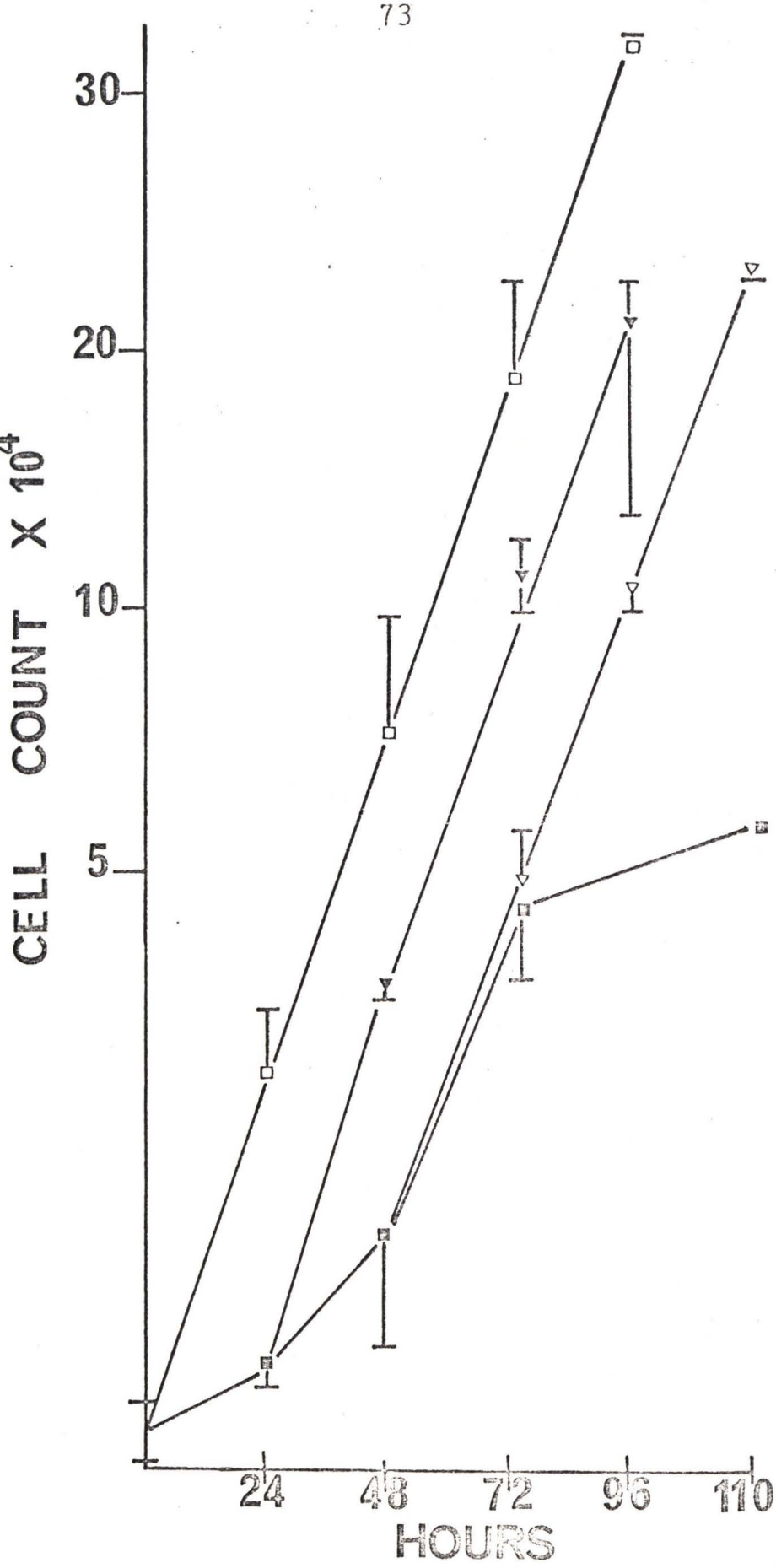

30
20
10
5
CELL COUNT X 10^4
24
48
72
96
110
HOURS

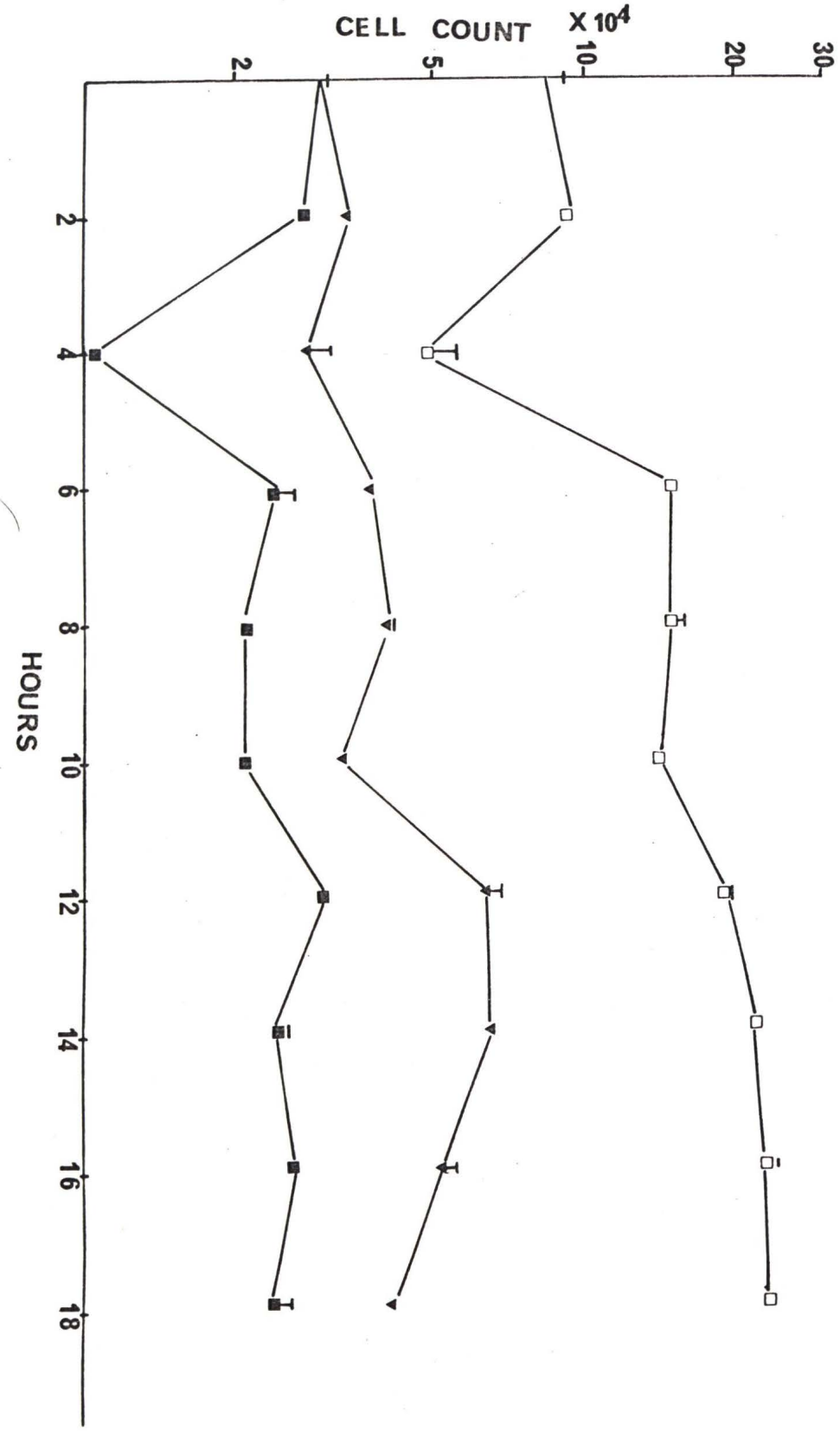
Figure 4. Cell synchrony induced by incubating in partially depleted medium for 24 hours. Cells were incubated for 24 hours before immersing in normal medium. A cell count of adhering cells was performed every two hours.

□ Control

■ Cells incubated in depleted medium

▼ Cells in normal medium after incubating in depleted medium for 24 hours

Error bars represent one standard deviation.


CELL COUNT X 10⁴
2
5
10
20
30
HOURS
2
4
6
8
10
12
14
16
18

synchronize the cells in G 1 of the life cycle. Such treatment should synchronize cells when immersed in normal medium. Cells were grown to confluence and distributed to tissue culture dishes containing normal medium for 24 hours before immersing in AMS medium. After an additional 24 hour incubation in AMS medium, normal medium replaced the AMS medium in half the experimental plates. The control plates were replenished with normal medium at the 24 and 48 hours.

When cells divide they detach from the tissue culture dish. In the experiment only adhering cells were counted so that a dip in cell count corresponded to cell division Figure 4. Unfortunately growing cells to confluence served to synchronize them. The doubling time from this graph was 14 hours corresponding to the difference between every second peak. In the control evidence of two dips suggested that control cells were synchronized in G1 and G2. In the control synchrony was lost at 18 hours. Cells incubated for 24 hours in AMS medium before immersing in normal medium, were still synchronized. Synchrony in experimental cells immersed in normal medium was evident at 18 hours. Confluence in tissue culture dishes was reached at over 3×10^{6} cells eliminating this as an explanation for the loss of synchrony in the control.

The difference between peaks should be equivalent to time for S, G2 and mitosis. This accounted for 6 hours. G1, therefore was 8 hours as previously reported. Most cell

division occurred 10 hours after replenishing cells in AMS medium with normal medium, implying that most of the cells were resting in G1 (84%). Why such a drastic decrease in attached cells should appear at 4 hours in the AMS control was unaccounted for. A concomitant increase in cell number was not observed. One can conclude that AMS treatment maintained cell synchrony. Reduction in cell counts could not be attributed to loss of viability, judged to be near 100% in all cases.

If the increase in cell proliferation were due to excessive amounts of exogenous nutrients or the supply of necessary nutrients *via* cell death, then the recovery of a normal doubling time should be a function of cell density. When this experiment was performed, restoration was faster at lower cell density Figure 5. An initial cell count of 76.4×10^4 in AMS medium had a doubling time of 96 hours by extrapolation. An initial count of 23×10^4 cells had a doubling time of 72 hours. This would suggest that cell proliferation was retarded by a nutritional deficiency in the AMS medium. It might also suggest that cells metabolize the ingredients essential to proliferation Williams (1975).

The next experiments were performed to ascertain which part of the AMS extraction procedure was responsible for this effect. Table 2 demonstrated that milliporing the medium did not eliminate the serum components essential to cloning. Dialysis severely retarded cell division Figure

Figure 5. Cell division in partially lipid depleted medium as a function of cell number

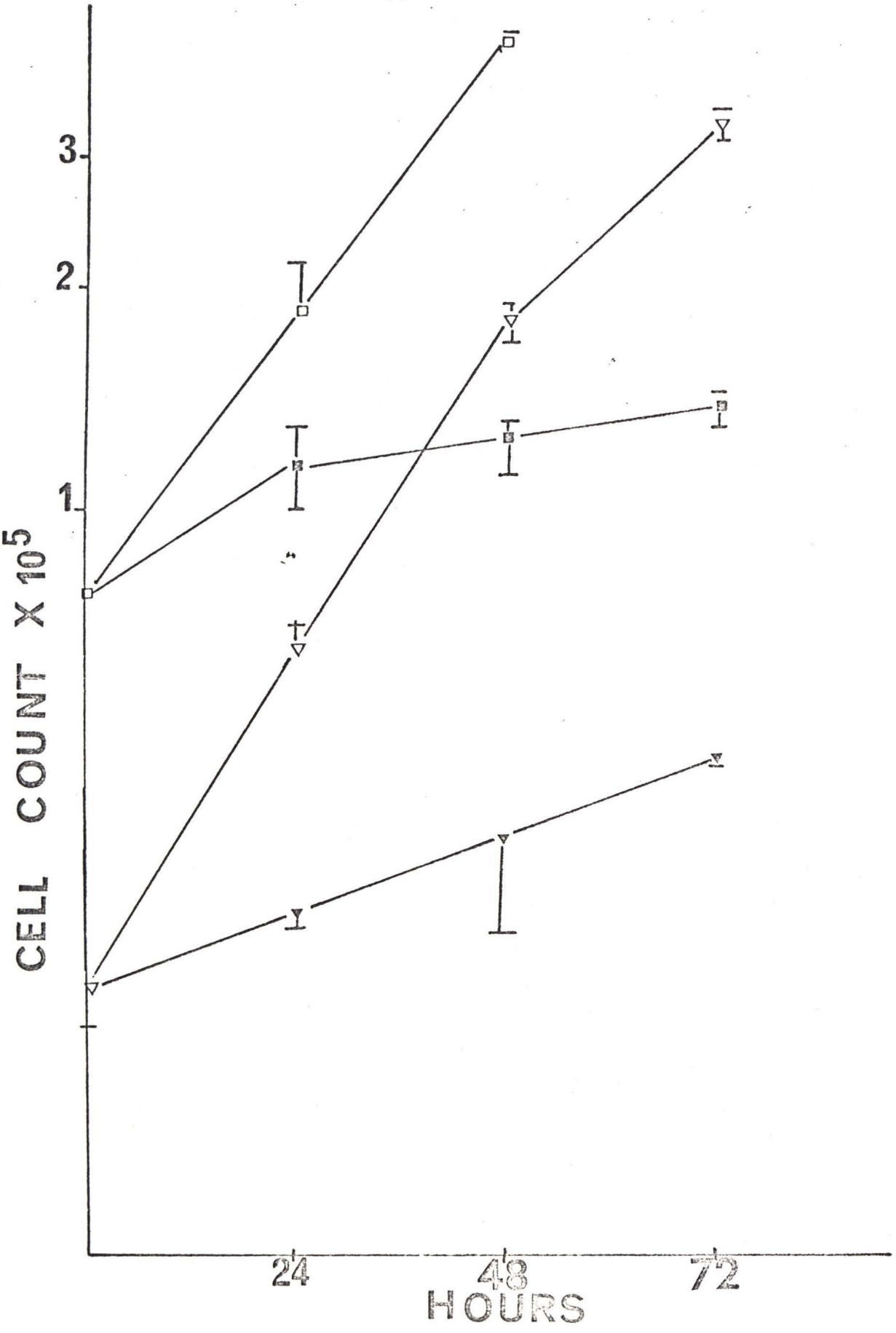
□ Control

■ High initial cell number in depleted medium

▽ Control

▼ Low initial cell number in depleted medium

Error bars represent one standard deviation.


CELL COUNT X 10^5
3
2
1
24
48
72
HOURS

6. Normal serum was included in the dialysis of AMS Batch 9. Another batch of normal serum was dialysed against serum-free CTM with no traces of ammonium sulfate. From Figure 6 one could infer that dialysis itself severely reduced calf proliferation. Addition of the dialysate from normal serum to AMS serum in concentrations equivalent to that found in normal medium, failed to restore normal division. This implied that ammonium sulfate treatment compounded by dialysis, was responsible for reducing cell division time Figure 7. This was also supported by Figure 8 when AMS supernatent, extensively dialysed to remove ammonium sulfate, was added to AMS serum. Cell division was again reduced.

Dialysis has been reported to deplete serum of its lipids Spector (1969); Henning (1970). Since amino acids, vitamins, and glucose were supplied by Eagle's medium and the protein panorama shown by electrophoresis (Appendix I) was conserved, one might infer that lipid depletion was responsible for the reduction in cell division. If cell proliferation were due to protein denaturation, then recovery of cell division in AMS medium should be similar from AMS batch to batch because the ammonium extraction procedure and final protein concentration were unchanged. If decreases in cell proliferation were explained by lipid depletion, recovery in AMS medium should be a function of lipid depletion. Figure 9 and Appendix I support the latter

Figure 6. Cell division in dialysed serum

□ Control

▽ Cells in medium with serum dialysed against medium without serum

▼ Cells in medium with serum dialysed as a control in Ammonium sulfate extraction

Error bars represent one standard deviation.

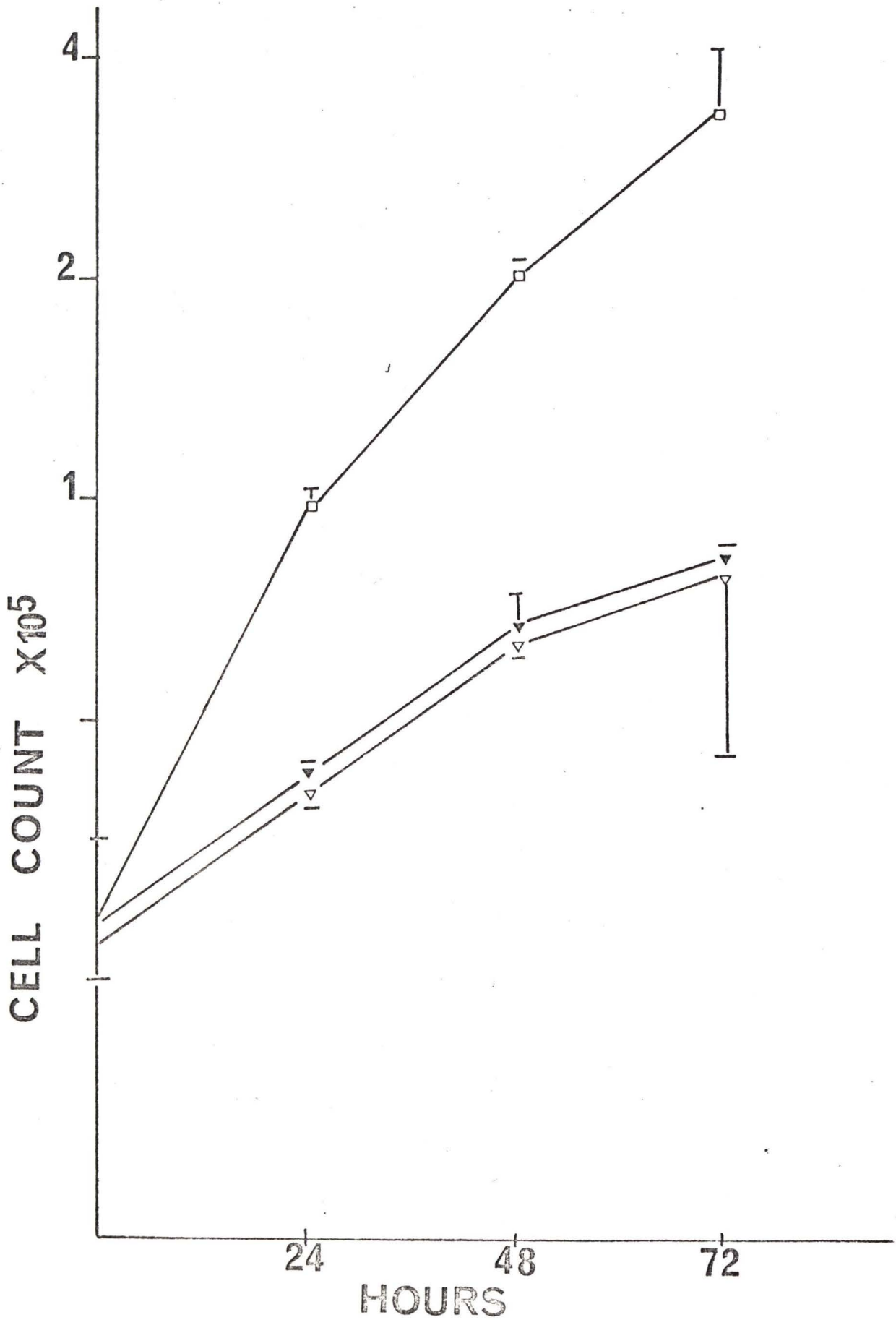

4
2
1
CELL COUNT X10⁵
24
48
72
HOURS

Figure 7. Cell proliferation in partially lipid depleted medium with dialysate from normal serum.

□ Control

▼ Control in partially depleted medium

■ Cells in depleted medium plus normal dialysate

Error bars represent one standard deviation.

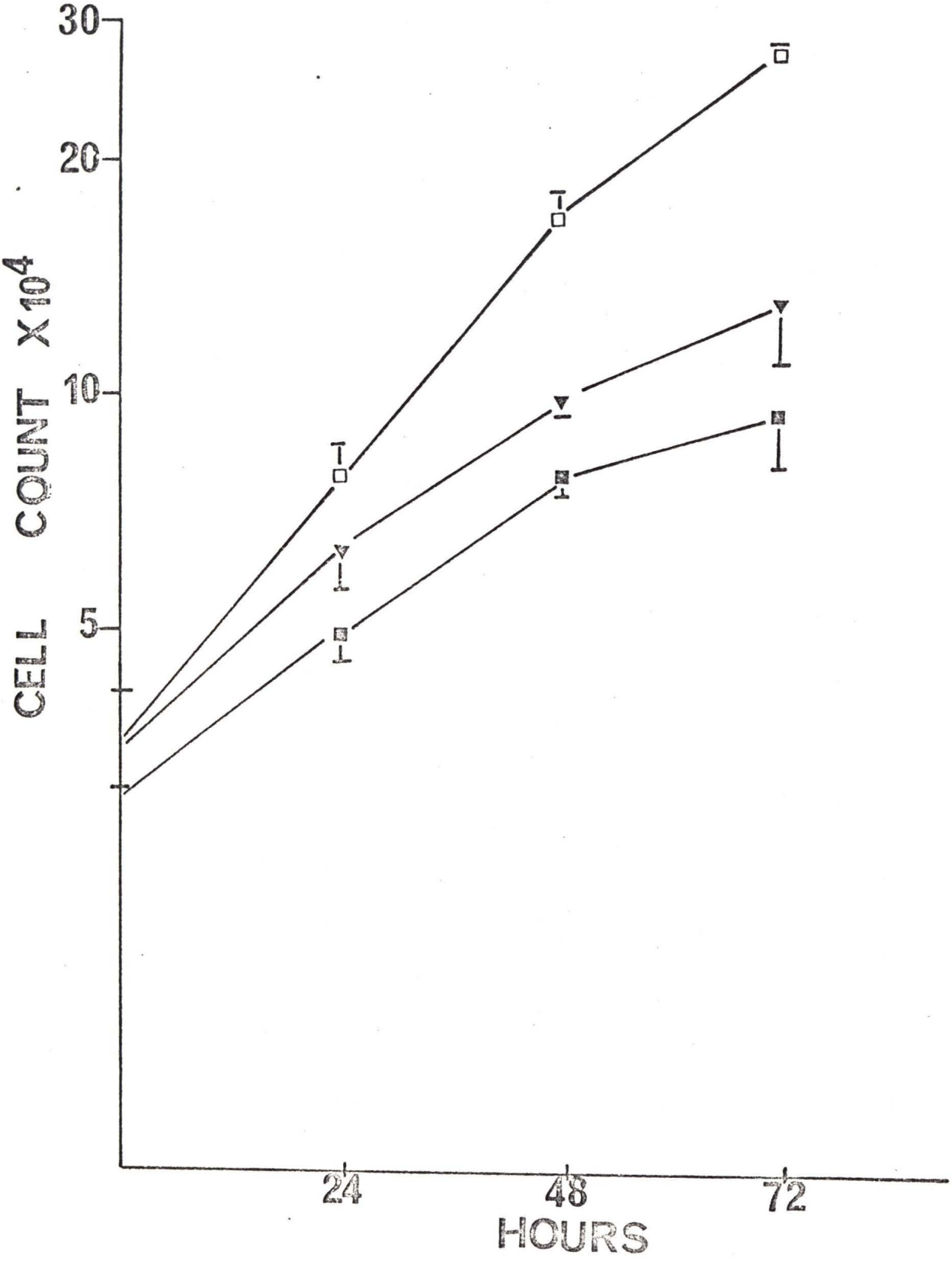

30
20
10
5
CELL COUNT X10^4
24
48
72
HOURS

Figure 8. Cell proliferation in partially lipid depleted medium and ammonium sulfate dialysate

□ Control

▽ Control in depleted medium

■ Cells in depleted medium plus dialysate

Error bars represent one standard deviation.

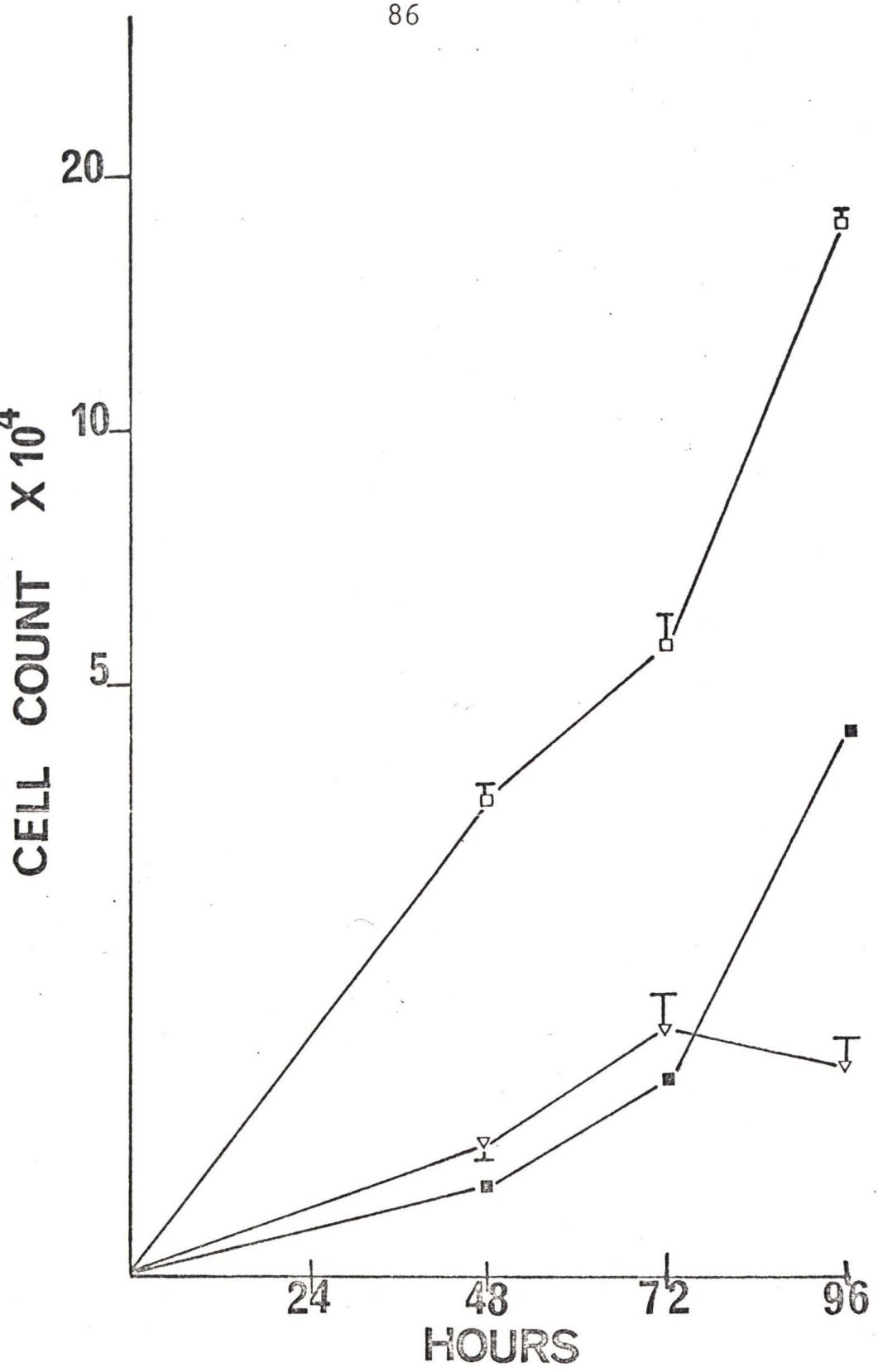

20
10
5
CELL COUNT X 10^4
24
48
72
96
HOURS

Figure 9. Cell proliferation in different batches of depleted medium.

□ Control

▲ Batch 9

o Batch 10

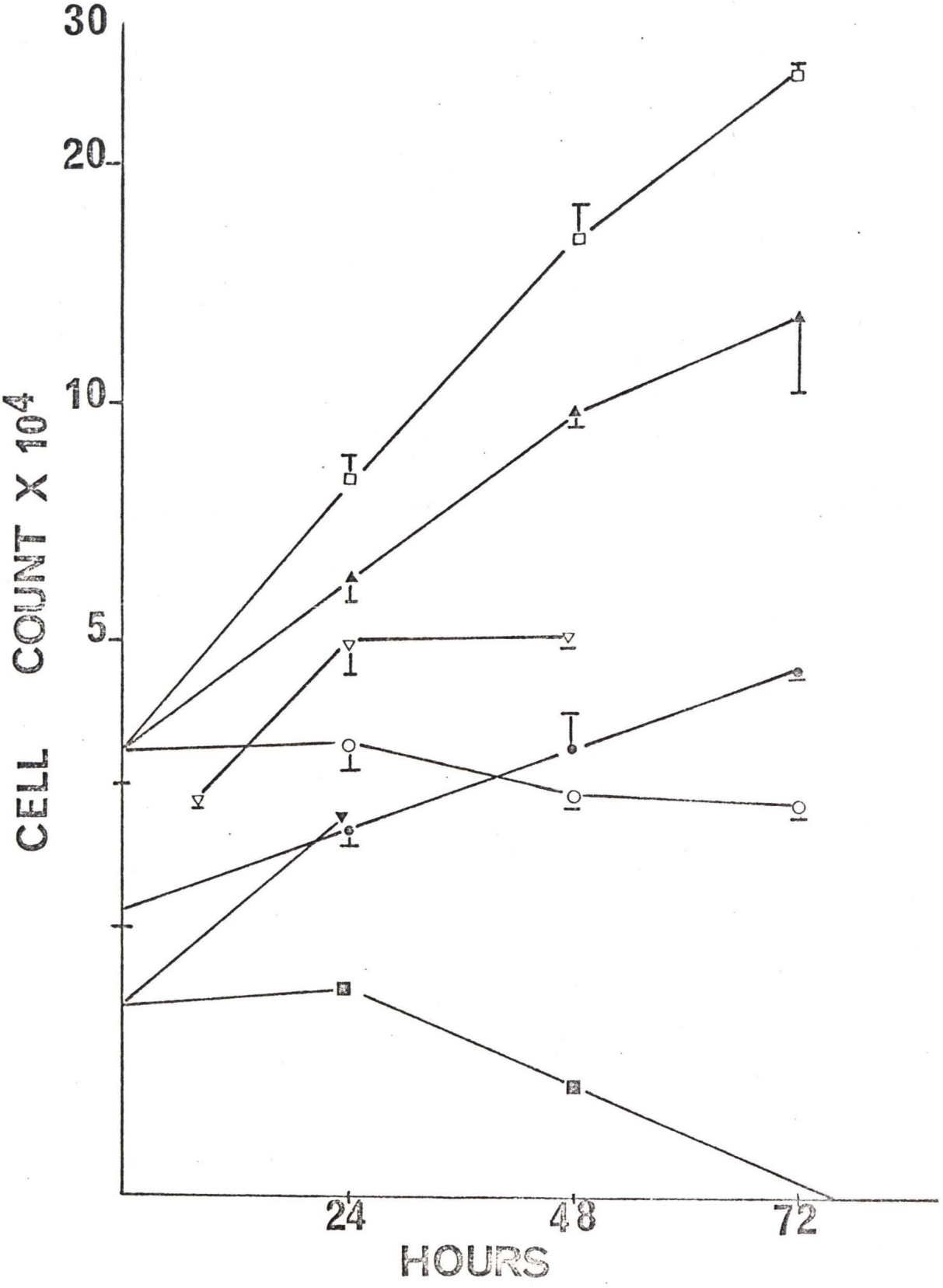
▽ Batch 8

▼ Batch 6

● Batch 4

■ Batch 3

Error bars represent one standard deviation.


30
20
10
5
CELL COUNT X 10^4
24
48
72
HOURS

postulation. The results from ethanol/ether treatment discussed in the next section implicated linoleate as the essential lipid component as previously reported by Ham (1963).

Ethanol/Ether Treatment

Lipid depleted medium was prepared according to Horowitz (1974) without denaturing the protein Horowitz (1977). Electrophoretograms, in addition to confirming this observation, indicated no preferential protein selection had occurred. Appendix I.

Cells were grown in normal medium to facilitate attachment. Bailey (1973) found attachment was impaired in lipid free medium. In Chinese hamster cells, plating efficiency was reinstated when linoleate supplemented serum-free medium. The reversibility of effects from incubation in lipid free serum in halting cell proliferation, was implied by Figure 10. Replenishing with normal medium was correlated with restoration of cell division. Data from other cell types suggested that proliferation was suspended in G 1 of the life cycle Holley (1974); Cornell (1976); Hatten (1976) Figure 4.

Figures 11 and 12 supported previous data from Ham in 1963 that linoleate was the essential missing ingredient in lipid free medium since palmitic acid was not effective in restoring cell division. The reversibility of avidin, as reported by Horowitz (1974) and Wizneiski (1973) with L

Figure 10. Cell proliferation in normal medium after a 24 hour incubation in lipid depleted medium

- ◻ Control
- ■ Control in depleted medium
- ▼ Cells in normal medium after incubation in depleted medium

Error bars represent one standard deviation.

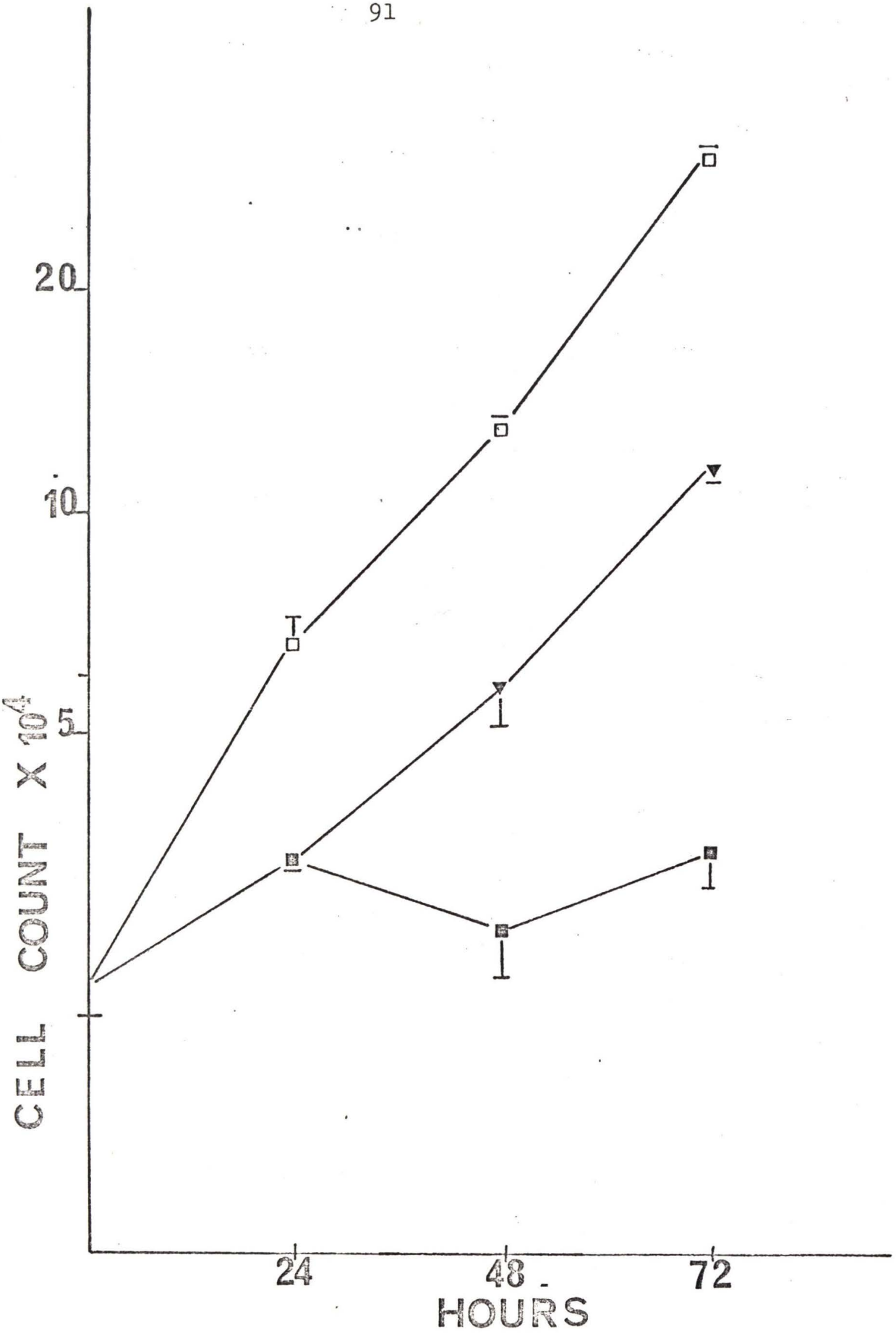

20
10
5
CELL COUNT X 10⁴
24
48
72
HOURS

Figure 11. Cell proliferation in lipid free medium supplemented with linoleate or linoleate plus avidin.

□ Control

○ Linoleate in lipid free medium

● Linoleate plus avidin in lipid free medium

■ Lipid free medium

Error bars represent one standard deviation.

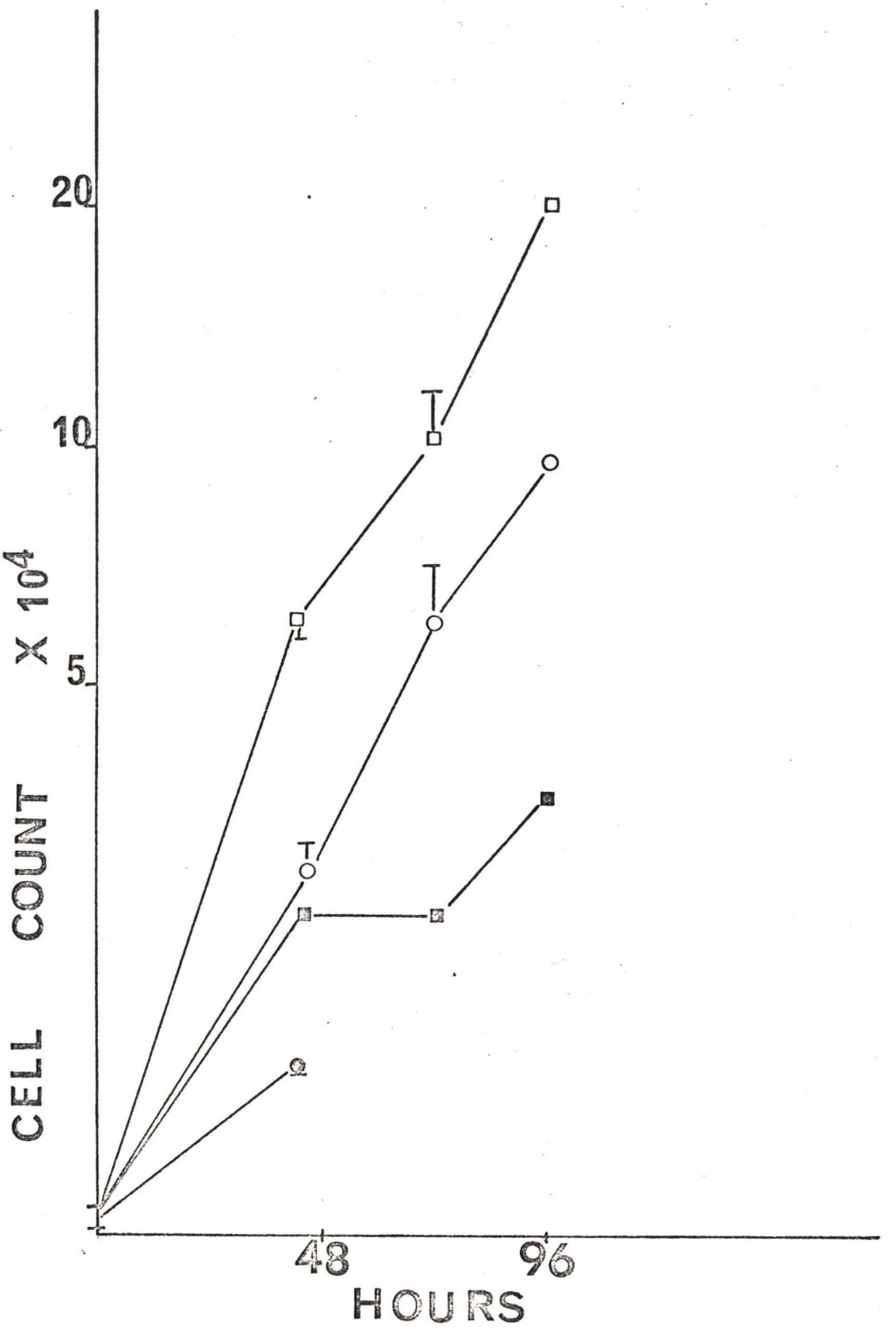

20
10
5
CELL COUNT X 10^4
48
96
HOURS

Figure 12. Cell proliferation in lipid free medium supplemented with palmitic acid or linoleate plus avidin in lipid free medium.

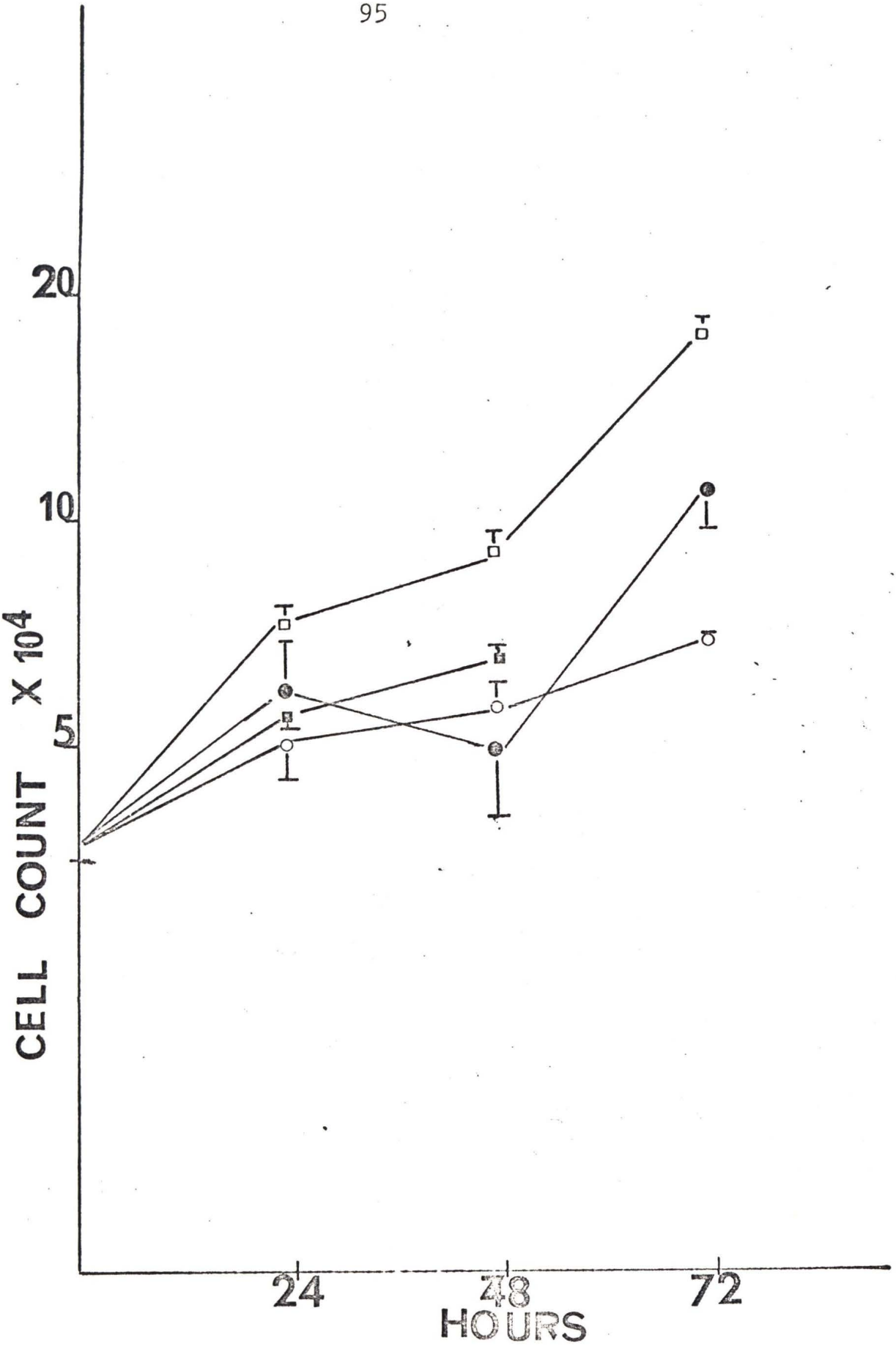
□ Control

○ Palmitic acid

● Linoleate plus avidin

■ Lipid free medium

Error bars represent one standard deviation.


20
10
5
CELL COUNT X 10^4
24
48
72
HOURS

cells, was also confirmed here by assaying cloning efficiency Table 4. A comparison between reinstatement of cell proliferation with linoleate and with linoleate plus avidin supplementation would suggest that "*de novo*" synthesis was also necessary in restoring normal cell division since avidin reduced normal cell division. These data also implied that any protein damage was not detrimental to cell proliferation or that such damage was soon repaired. Avidin binds biotin which in turn decreases "*de novo*" fatty acid synthesis Wizneiski (1973); Horowitz (1974); Green (1975).

A change in unsaturated/saturated fatty acid composition has been reported to affect a myriad of cell functions such as membrane fluidity, phase transitions, cell permeability, lectin-induced concanavalin binding and agglutinability, endocytosis, lateral diffusion of cell surface antigens, viral absorption, cell/cell and cell virus fusion, decreased cell/cell adhesion, membrane enzyme activity and differentiation Prives (1977); Horowitz (1974; 1977); Maloney (1978); Li (1975) Wisneiski (1973); De Gier (1968); Engelhardt (1976); Schroeder (1976); Monard (1977).

Bailey (1973) and Wizneiski (1973) found incubating in lipid free medium tended to increase the degree of saturation in phospholipid fatty acids. Growing 3T3 and SV 3T3 cells in lipid free medium plus biotin failed to alter the unsaturated/saturated fatty acid ratio Horowitz (1974).

With macrophages Maloney (1977) reported increased membrane fatty acid saturation after incubating in serum free medium. The cell/cell adhesion test suggested that CHO cells experienced greater fatty acid unsaturation in the membrane compared to normal cells. Figure 13. This test did not accurately assay membrane fatty acid unsaturation. Adhesion was decreased when the degree of fatty acid unsaturation was increased or when saturated short chain fatty acid incorporation was increased Curtis (1975).

Several authors have altered the saturated/unsaturated fatty acid ratio in the plasma membrane. Horowitz (1976) reported that cells preferentially use an exogenous fatty acid supply and that such substitution can be enhanced by adding avidin to complex biotin, and long chain saturated fatty acids. Both inhibit *de novo* fatty acid synthesis. Such alterations were maximized when incubation was for 24 hours or less.

Both Ferguson (1975) and Williams (1974) with LM cells reported linoleate supplementation did not alter the unsaturated/saturated fatty acid ratio. Linoleate substitution was maximized within 24 hours in LM cells. Longer incubation led to longer chain unsaturated fatty acid incorporation. Glasser (1974) also reported that linoleate supplementation led to slightly increased phosphatidylcholine concentrations. Schroeder (1976) also reported a decrease in plasma membrane sterol. Membrane fluidity has

TABLE IV

Ability to Recuperate as Ascertained by Cell Count, Cloning Efficiency and Cell Morphology After Incubating for 24 hours in Lipid Free Medium with Supplements

Treatment	Cell Count X 10^5	Cloning Efficiency %	Morphology
Control	57.3	61.3	
S.D.	1.89	0.14	
Linoleate	28.4	41.2	lipid droplets
S.D.	1.59	5.00	
(L)			
Linoleate plus avidin	31.6	49.8	lipid droplets
S.D.	3.00	3.90	
Lipid free	28.7	43.9	
S.D.	1.77	3.11	

Original Cell Count 33.5×10^4

been altered with linoleate supplementation as reported by Prives (1978); Hoover (1977) and Kasai (1976). Linoleate supplementation at a concentration of 4×10^{-5}M with avidin in these experiments were conducted for 25 hours. Bailey (1973) and Ferguson (1975) reported that linoleate supplementation was accompanied by accumulation of lipid droplets as observed in the cloning experiments. The other method used to ascertain plasmalemmal substitution was the cell/cell adhesion test as described by Hoover (1977) Figure 13.

Palmitic acid supplementation increased the ratio of saturated fatty acids in the cellular phospholipids of cells provided the amounts used were less than 100 ug/ml of medium. Above this concentration the saturated/unsaturated fatty acid ratio approached that the control Williams (1974); Wizneiski (1973); Horowitz (1974). In Tetrahymena pyriformis, Kasai (1976) suggested that when membrane saturation exceeded a threshold, membrane desaturases were activated to restor original ratio. In the following experiments a 2×10^{-5}M concentration of palmitic acid was used in lipid free medium. Cells were incubated in this medium for 24 hours before cooling to -196°C at various rates. Unfortunately no evidence exists for the success of this treatment in altering membrane fluidity.

When cholesterol-free lipoprotein was incubated with erythrocytes, the erythrocyte membrane cholesterol/

phospholipid ratio was decreased to one half after one day. DMSO (10%) amplified the depletion further Bruckdorfer (1976). Similar behavior has been observed in mouse fibroblasts, L cells, and macrophage as reported by the same review. Membrane cholesterol, as assayed by biochemical and enzymatic assays, had decreased. The reduction was probably not as pronounced as in other cells since Chang (1977) found that incubation of CHO cells in lipid free medium instigated cholesterol synthesis within two hours.

Cholesterol depletion has been correlated with resistence to osmotic shock and increased fluidity Jain (1975). Bruckdorfer and Graham (1976) suggested cholesterol depletion led to osmotic fragility. In all cells studied, with the exception of pig erythrocytes, cholesterol depletion was correlated with increases in permeability coefficient.

The permeability coefficent of a cell was the parameter of interest in these experiments. Increasing permeabiltiy consistent with a high unsaturated/saturated fatty acid ratio should predispose the cells to greater dehydration effects at slow cooling rates. Decreasing permeability *via* saturated membrane/unsaturated fatty acid supplementation should yield higher survival at slow cooling rates. If fluidity were a factor of major importance as suggested by work done by Raccah (1975) and Kruuv (1976;1978), then unsaturated fatty acid supplementation or cholesterol depletion should yield higher survival with freeze/thaw.

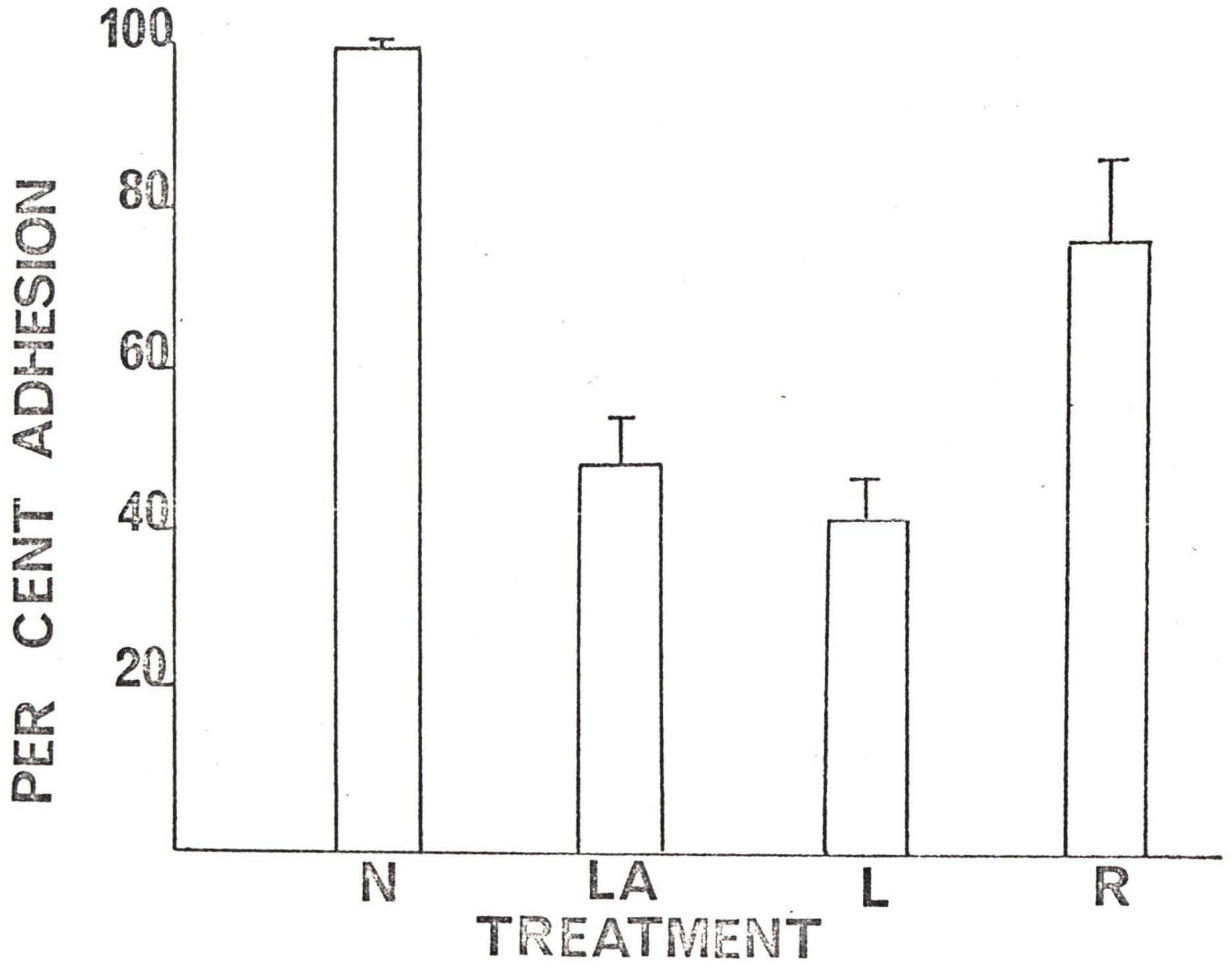
Figure 13. Cell/cell adhesion of cells pre-incubated in linoleate and linoleate plus avidin in lipid free medium. Error bars represent standard error.

N Control

LA Linoleate plus avidin

L Linoleate

R Lipid free


PER CENT ADHESION
100
80
60
40
20
N
LA
L
R
TREATMENT

Serum Cryoprotection

The following experiments were designed to investigate the cryoprotective effects of lipids in serum during freezing and thawing at slow cooling rates. The analog to serum in other systems was egg yolk. In the latter, thermal shock protection was correlated with lipoprotein purification. Complete purification resulted in a loss of cryoprotection implicating protein or lipid components Pace (1975). Morris (1975) found that phosphatidylserine a constituent of egg yolk, protected erythrocytes during freeze/thaw.

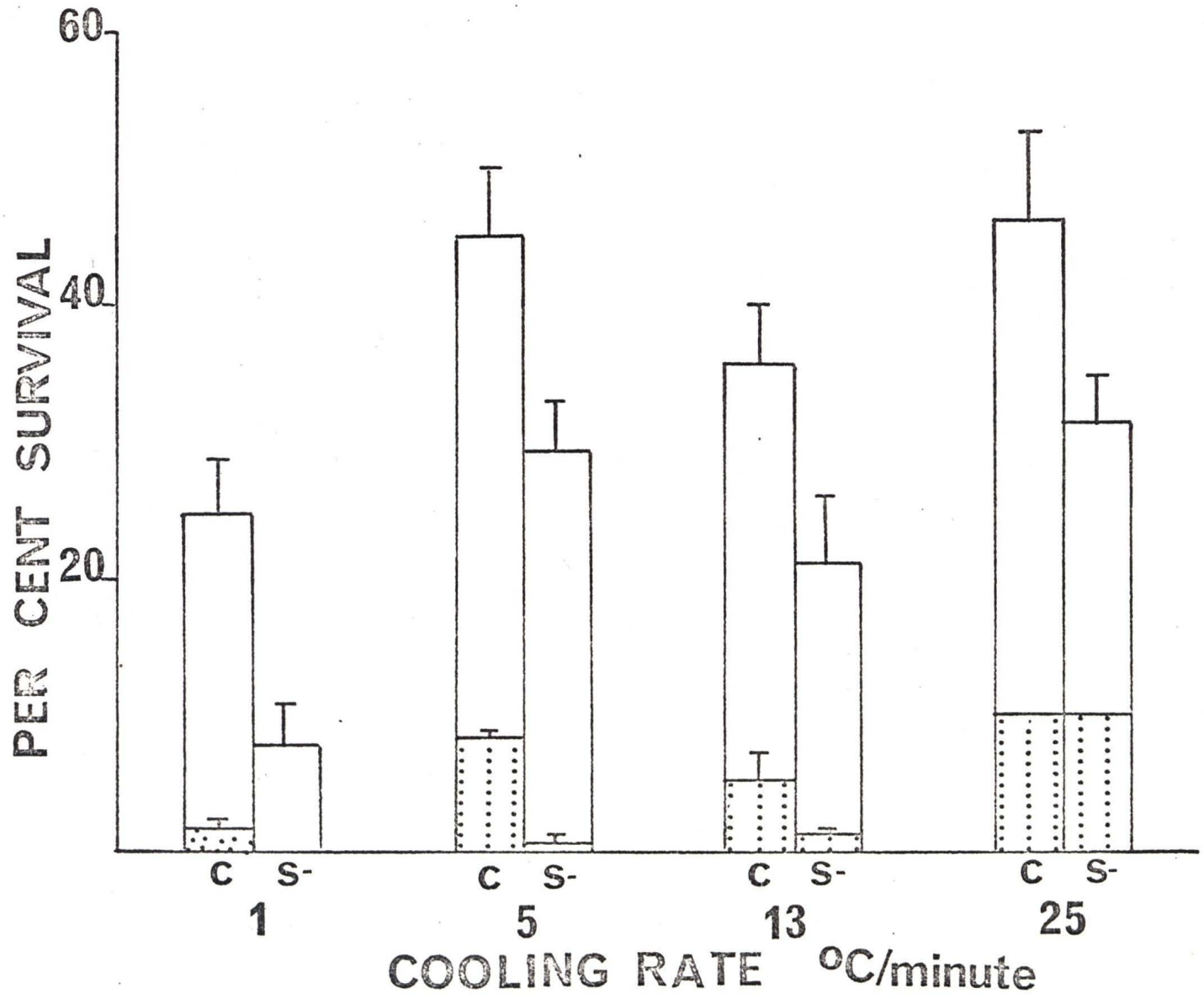
If phospholipids were implicated in serum protection, a relationship between freeze/thaw and thermal shock might be inferred. Both ethanol/ether and ammonium sulfate precipation treatments were designed to deplete the serum without extensive protein denaturation. The medium was prepared to conserve protein concentration found in normal medium. If the lipid constituent were cryoprotective, then loss of protection should be closely correlated with lipid depletion.

Figure 14 demonstrated the protection from DMSO and serum at cooling rates of 1°C, 5°C, 13°C and 25°C/minute. Control cells were frozen in 10% fetal calf serum plus or minus DMSO. S- represented normal cells frozen without serum. The DMSO concentration used in all experiments was 2.5%. All points were significantly different from each

Figure 14. Freeze/thaw survival of normal cells frozen at various rates in serum free medium plus or minus 2.5% DMSO. Results for DMSO minus control at 25°C/minute are from W. Connor's thesis (1973). Shaded areas represent cells frozen without DMSO. Error bars represent one standard deviation.

c Control

s- Serum free medium


60
40
20
PER CENT SURVIVAL
C
S-
1
5
13
25
COOLING RATE °C/minute

other at less than the 5% probability as determined by the "Students" t-Distribution".

Two survival optima were demonstrated for both frozen/thawed control and serum free samples, provided DMSO was included in cooling medium. Data from control cells frozen without DMSO, although quite inclusive, implied that the double optima might be a cell specific effect rather than a DMSO artifact. These data confirmed an earlier study on CHA lung fibroblasts frozen in 0.3 M DMSO. This paper also reported that intracellular ice formation occurred at 50°C/minute Mazur (1972). This would suggest that there might be an additional physical/chemical factor to be evaluated or that cell survival was peculiar to cooling rate used.

Figure 15 demonstrated the protective effect of lipid depleted medium on freezing and thawing survival of normal cells. AMS 8 and ethanol/ether sera were used in these experiments. Complete lipid depletion (E) when compared to AMS 8 and serum-free medium, yielded significantly higher survival at 1°C/minute. At 5°C/minute and 13°C/minute, survival was significantly lower in lipid free medium from in other treatments. At 25°C/minute survival in lipid free medium was similar to that found in serum free medium and partially depleted medium (AMS8).

Partially lipid-depleted medium (AMS8) yielded recovery equivalent to that found in serum-free medium at 1°C, 13°C,

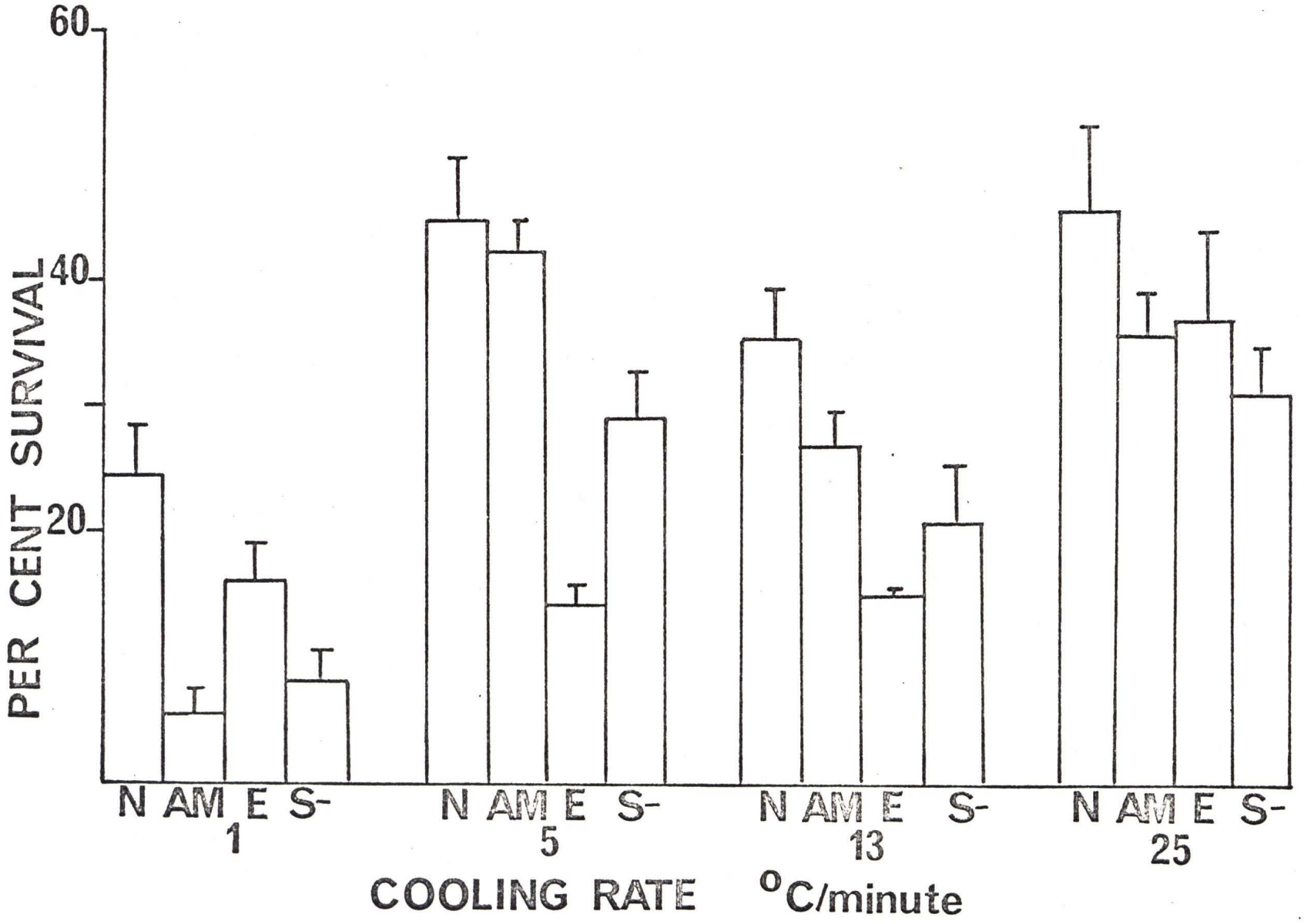
Figure 15. Freeze/thaw survival of normal cells cooled at various rates in lipid free, partially lipid depleted (Batch 8), and serum free medium with 2.5% DMSO. Error bars represent one standard deviation.

N Control

AM Partially depleted medium

E Lipid free medium

S- Serum free medium


60
40
20
PER CENT SURVIVAL
N AM E S-
1
N AM E S-
5
N AM E S-
13
N AM E S-
25
COOLING RATE °C/minute

and 25°C/minute. Survival at 5°C/minute was similar to that in normal CTM. Therefore, with the exception of 5°C/minute in partially depleted serum, lipid depletion lowered survival significantly at all rates studied.

Knight (1972) with lymphocyte freeze/thaw recovery found that decreasing the DMSO concentration from 10% to 5% served to broaden the distance between optimum cooling rates. Comparing CHO data of Harris(1974) when cells were frozen in 10% DMSO to that of Mazur (1972) when cells were frozen in 0.3 M DMSO (2.5%) also demonstrated this. This would explain why lipid depleted serum (E) yielded significantly higher survival from partially depleted at 1°C/minute. The same effect, however, should be shown with cryprotection from serum-free medium. Only at 25°C/minute did complete lipid depletion effectively correlate with serum removal.

It may be that at slower cooling rates lipid depletion did not wholly account for serum protection. If the protein fraction accounted for protection, then freeze/thaw survival in lipid depleted medium and partially depleted medium should not be significantly different from normal medium. Only at 5°C/minute after cooling in partially-depleted medium was this true Figure 15. Another explanation for the difference between serum free medium and lipid free medium cryoprotection was that the non-serum constituents in serum free

medium were slightly more concentrated (10%) than in other media. Neither water or isotonic salt were added to dilute constituents to normal concentrations. The addition of water would introduce hypotonic shock and addition of salt has been shown to alter freeze/thaw survival in other cell types Calcott (1975); Woolgar (1974). Adjusting the osmolarity with sugar was not a satisfactory procedure because sugars were cryoprotective. It may be that non-serum constituents were responsible for freeze/thaw disparities between lipid free and serum free medium.

Generally a cryoprotectant has been shown to behave in predictable ways as concentration and cooling rate were varied. This has been previously reviewed (Section III). High concentrations of cryoprotectant should shift the optimum to slower cooling rates. When survival was plotted against concentration at one cooling rate, recovery should reach a plateau. Survival should decrease at very high concentrations. Data from Thorpe (1976) suggested that at serum concentrations exceeding 15%, freeze/thaw lymphocyte recovery was diminished.

Secondly when the optimum cooling rate was exceeded the protective effect of serum was decreased. Freeze/thawing recovery of CHO cells in partially depleted serum at 13°C/minute and 25°C/minute confirmed this Figure 15.

Reference to Appendix I demonstrated the variability of ammonium sulfate treatment in depleting serum lipids. Normal cells were frozen in various batches of AMS serum supplemented with 2.5% DMSO. In figures 16, 17 and 18 freeze/thaw survival at 5°C/minute and 13°C/minute was plotted against organic phosphate, fatty acid, and total cholesterol concentration in these media. If one of the serum consitituents were responsible for serum cryoprotection, then increasing normal fetal calf serum concentrations to 20% should give freeze/thaw survival approximating one of these curves. Normal cells were frozen at 13°C/minute in 5, 10, 15, and 20% fetal calf serum in CTM with 2.5% DMSO. There was a close correlation between organic phosphate and % serum freeze/thaw survival Figure 19.

Reference to organic phosphate (Figure 16) demonstrated the expected plateau at 5°C/minute as was reported for lymphocytes at optimum cooling rate of 1.8°C/minue Thorpe (1976). These data also suggested that as cooling rate increased, the effectiveness of lipid protection decreased. Much higher concentrations of phospholipids would be needed to provide equivalent cryoprotection, if the concentrations were not yet cytotoxic.

These data implicated phospholipids in serum protection. Unfortunately, triglycerides were not discounted as a

Figure 16. Freeze/thaw survival at cooling rates of 5°C/minute and 13°C/minute in medium with different amounts of organic phosphate. The last point represents control. Bars = 1 Standard deviation

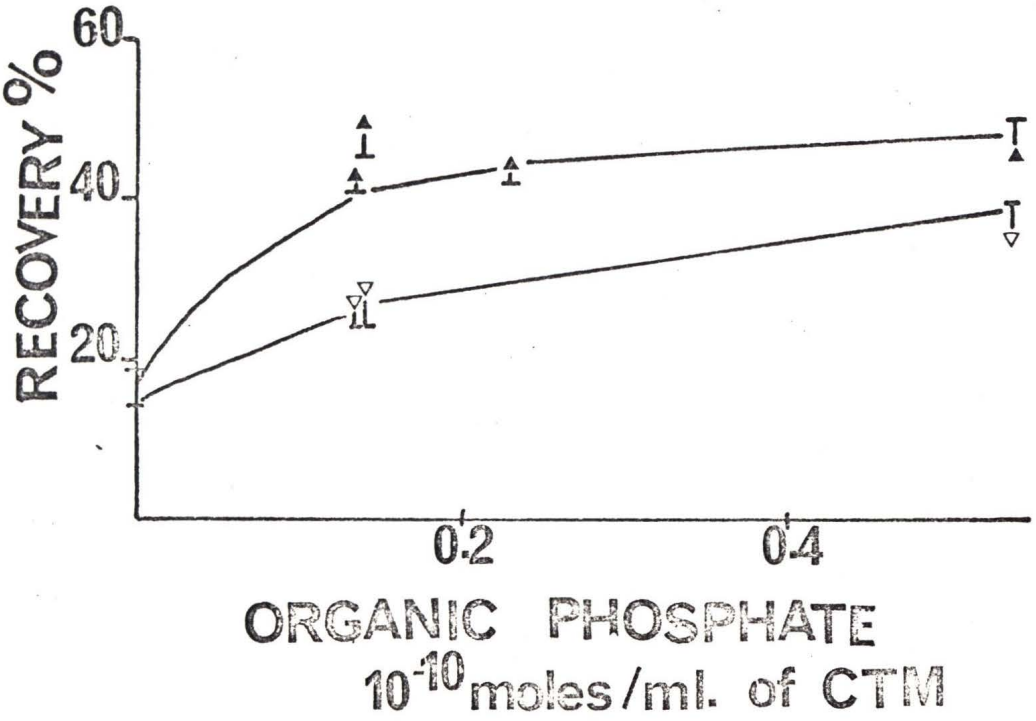
▲ 5°C/minute

▽ 13°C/minute

Figure 17. Freeze/thaw survival at cooling rates of 5°C/minute and 13°C/minute plotted against fatty acid concentrations in different batches of medium. Results at 0.54 μ moles/ml. of CTM represents the control. Bar represents one standard deviation.

▲ 5°C/minute

▽ 13°C/minute


RECOVERY %
60
40
20
0·2
0·4
ORGANIC PHOSPHATE
10^{-10} moles/ml. of CTM

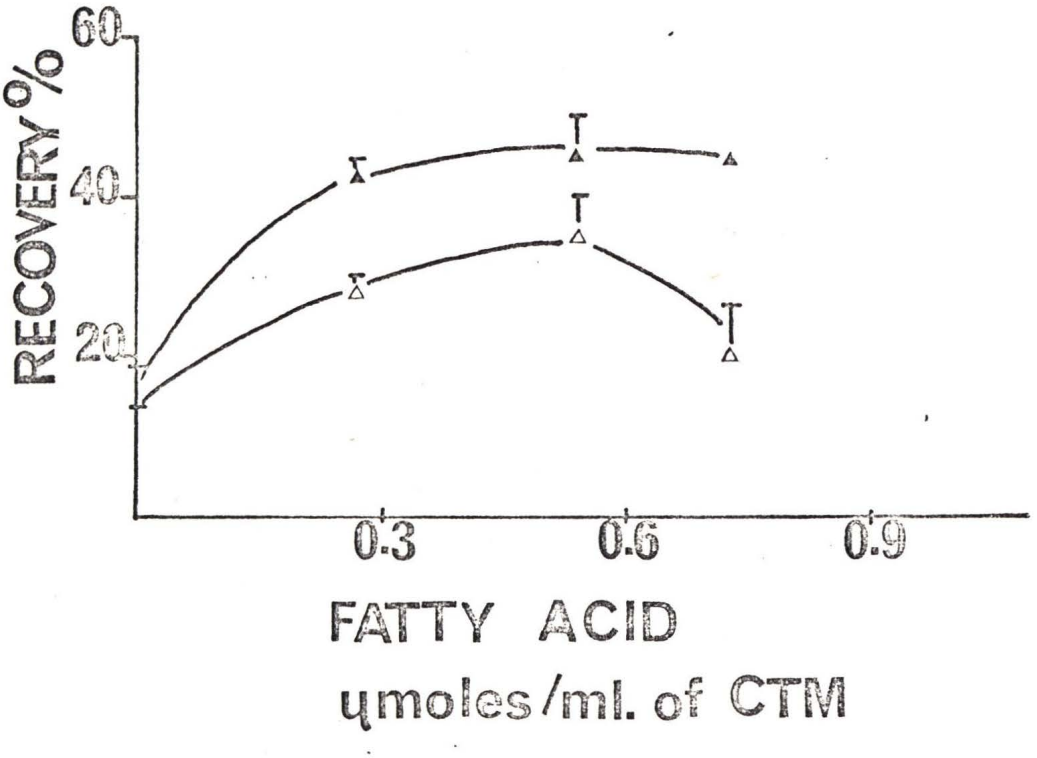
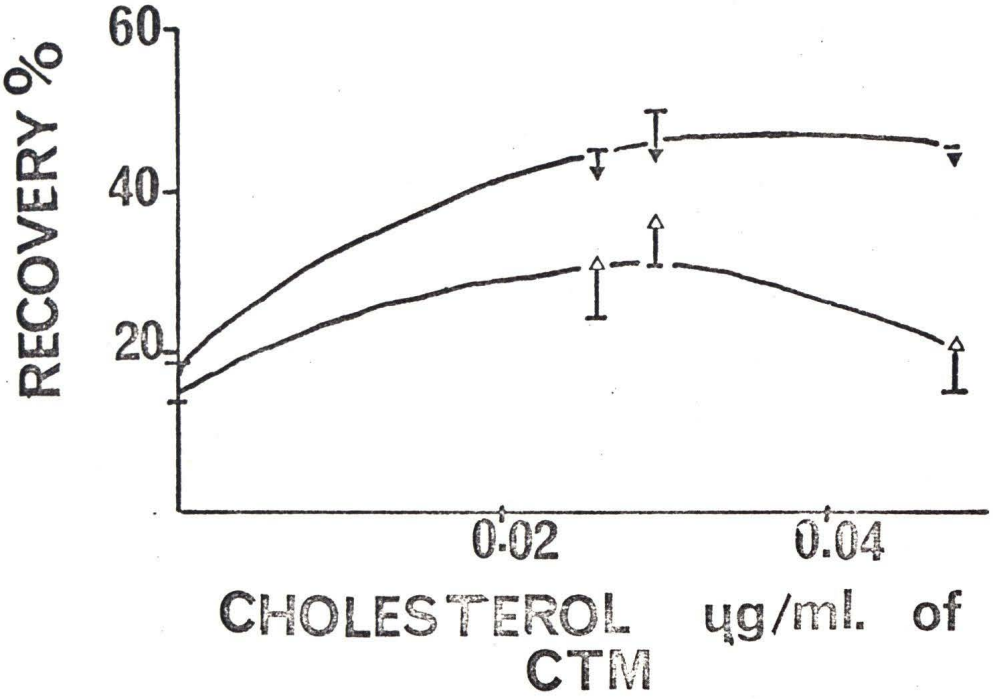

RECOVERY %
60
40
20
0·3
0·6
0·9
FATTY ACID
μmoles/ml. of CTM

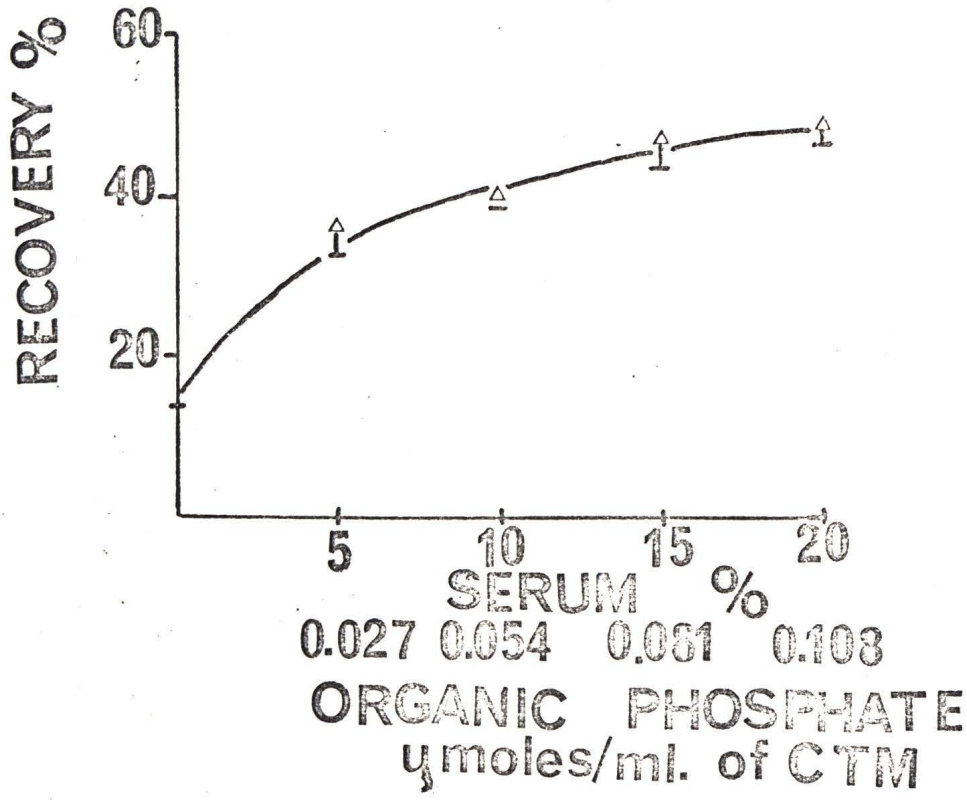
Figure 18. Freeze/thaw survival at cooling rates of 5°C/minute and 13°C/minute plotted against cholesterol concentration in different batches of medium. Results at 0.03 µg/ml. of CTM represent the control.

▼ 5°C/minute

Δ 13°C/minute

Figure 19. Freeze/thaw survival at 13°C/minute in medium with different amounts of serum. Results are also plotted against organic phosphate concentrations.


RECOVERY %
60
40
20
0.02
0.04
CHOLESTEROL µg/ml. of CTM


RECOVERY %
60
40
20
5
10
15
20
SERUM %
0.027
0.054
0.081
0.108
ORGANIC PHOSPHATE µmoles/ml. of CTM

potential source of protection. If glyceride inclusion contributed an additional source of protection, then phospholipid and serum protection might be expected to be significantly different. It was unlikely that glyceride depletion closely parallelled that of phospholipids. Because they were less miscible in water, one might expect them to be more tightly bound to the lipoprotein.

These data would suggest that the lipid moiety of the lipoprotein was important in serum protection. The lipid constituent implicated was phospholipid, although triglycerides have not been eliminated another as a source of cryoprotection. This might imply a relationship between thermal shock protection and freeze/thaw survival although such a speculation was discredited by G.J. Morris (1975).

Cholesterol

Experiments in Appendix II suggested that cells incubated in lipid free medium depleted their membrane cholesterol while those reared in AMS 8 accumulated excess membrane cholesterol. Normal cells had an intermediate amount of cholesterol.

Cells were grown for 24 hours in lipid free medium, normal medium, and AMS 8 medium before cooling at various rates in normal medium with 2.5% DMSO Figure 20. Cholesterol minus cells had significantly different results from normal cells at 1°C and 5°C/minute. At 25°C/minute results were significantly different at the 10% probability level. Cholesterol plus cells gave significantly different survival from normal cells at all cooling rates studied. Cholesterol plus results were significantly different from cholesterol minus cells at 1°C/minute and 25°C/minute. Therefore, the only significant freeze/thaw change occurred at 1°C/minute.

When cells were grown in AMS 8 or normal medium for 24 hours before cooling in AMS 8 medium supplemented with 2.5% DMSO, freeze/thaw recovery was not significantly different at 1°C/minute. At 5°C/minute and 13°C/minute survival was significantly lower in AMS 8 treated cells (cholesterol plus) Figure 21. This would lend support to the previous speculation that depleting serum and therefore cryoprotectant lipids should shift the optimum cooling rate to faster velocities. Freeze/thaw recovery in normal medium showed significant differences between these two treatments at all

Figure 20. Freeze/thaw survival in normal medium plus 2.5% DMSO of cells grown in lipid free medium, normal medium, and partially depleted lipid medium (Batch 8) for 24 hours. Bar represents one standard deviation.

C- Cells in lipid free medium. Cholesterol minus.

N Control

C+ Cells in partially depleted medium. Cholesterol plus.

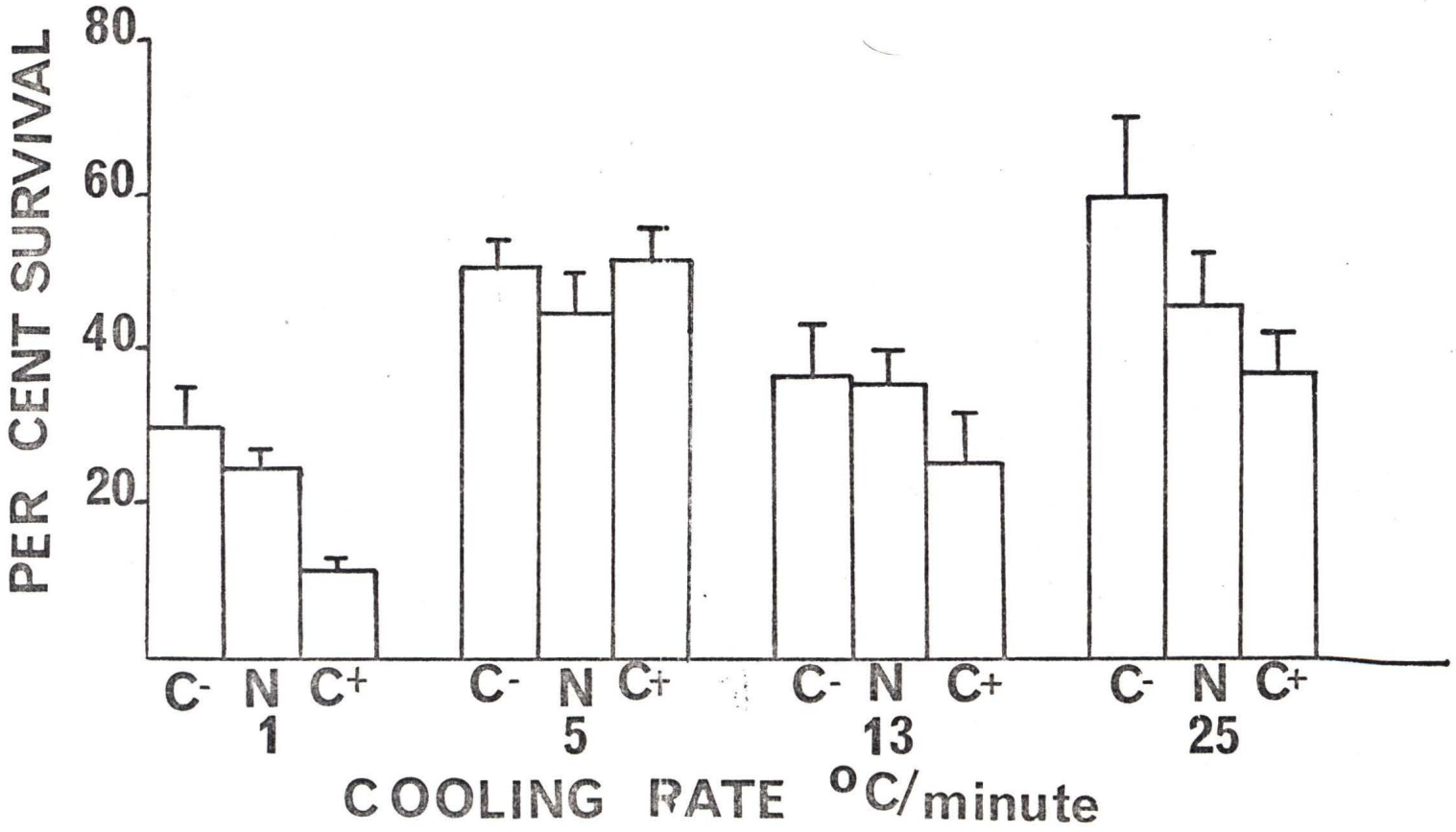
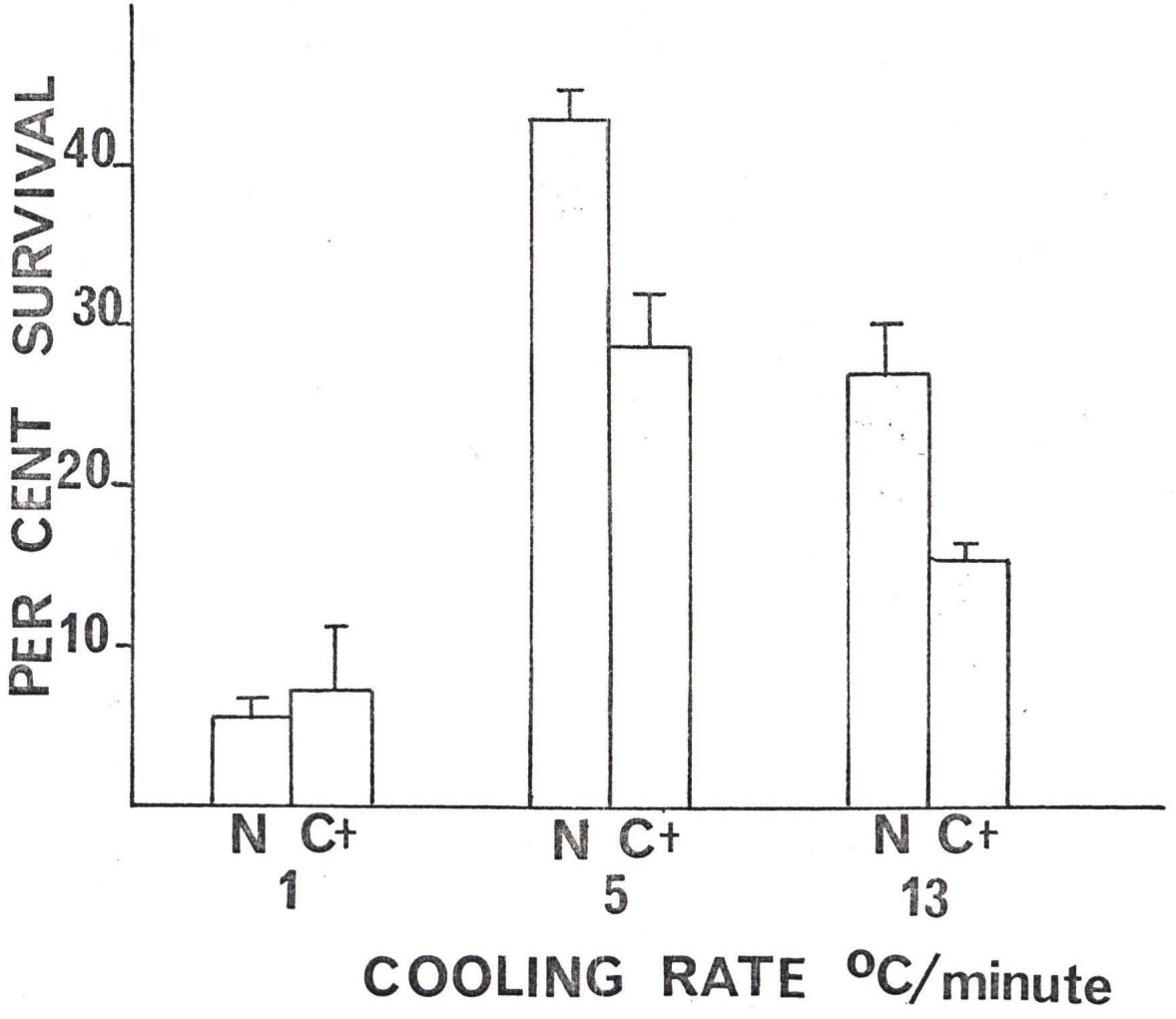

PER CENT SURVIVAL
80
60
40
20
C- N C+
1
C- N C+
5
C- N C+
13
C- N C+
25
COOLING RATE °C/minute

Figure 21. Freeze/thaw survival of cells grown for 24 hours in normal medium or partially depleted medium. Cells were frozen in partially depleted medium plus 2.5% DMSO. Bar represents one standard deviation.

N Control

C† Cells in partially depleted medium. Cholesterol plus.


PER CENT SURVIVAL
40
30
20
10
N C+
1
N C+
5
N C+
13
COOLING RATE °C/minute

cooling rates studied Figure 20.

A shift in significant freeze/thaw recovery differences between normal and lipid free serum did not occur.

When cells were grown in lipid free or normal medium before cooling in lipid free medium plus 2.5% DMSO, there were no significant differences in freeze/thaw recovery between the two treatments Figure 22. When cells were cooled in normal medium there was a significant difference in freeze/thaw survival at 1°C/minute Figure 20. This might imply a broadening difference between slow and fast optima. This was not tested. If this were true then a significant difference in freeze/thaw survival in lipid free medium should be found at even slower cooling rates.

When cholesterol plus cells and normal cells were frozen at various rates in medium without serum Figure 23, results were significantly different at 5°C/minute although there was a small but insignificant difference at 13°C/minute.

If a shift in the significance can be interpreted as a shift in the optimum cooling rate, then partially depleted medium, lipid free medium and serum free medium did indeed alter this rate Figure 20, 21, 22, 23.

Since cholesterol exchange from the cell to the medium occurred in DMSO, it would be interesting to see if this would account for serum protection. This was unlikely since the half life of cholesterol exchange in cells studied was about 4 hours Kirby (1977); Bruckdorfer (1976). The most

Figure 22. Freeze/thaw survival of cells grown for 24 hours in normal medium or lipid free medium and frozen in lipid free medium. Bar represents one standard deviation.

N Control

C- Cells grown in lipid free medium. Cholesterol minus.

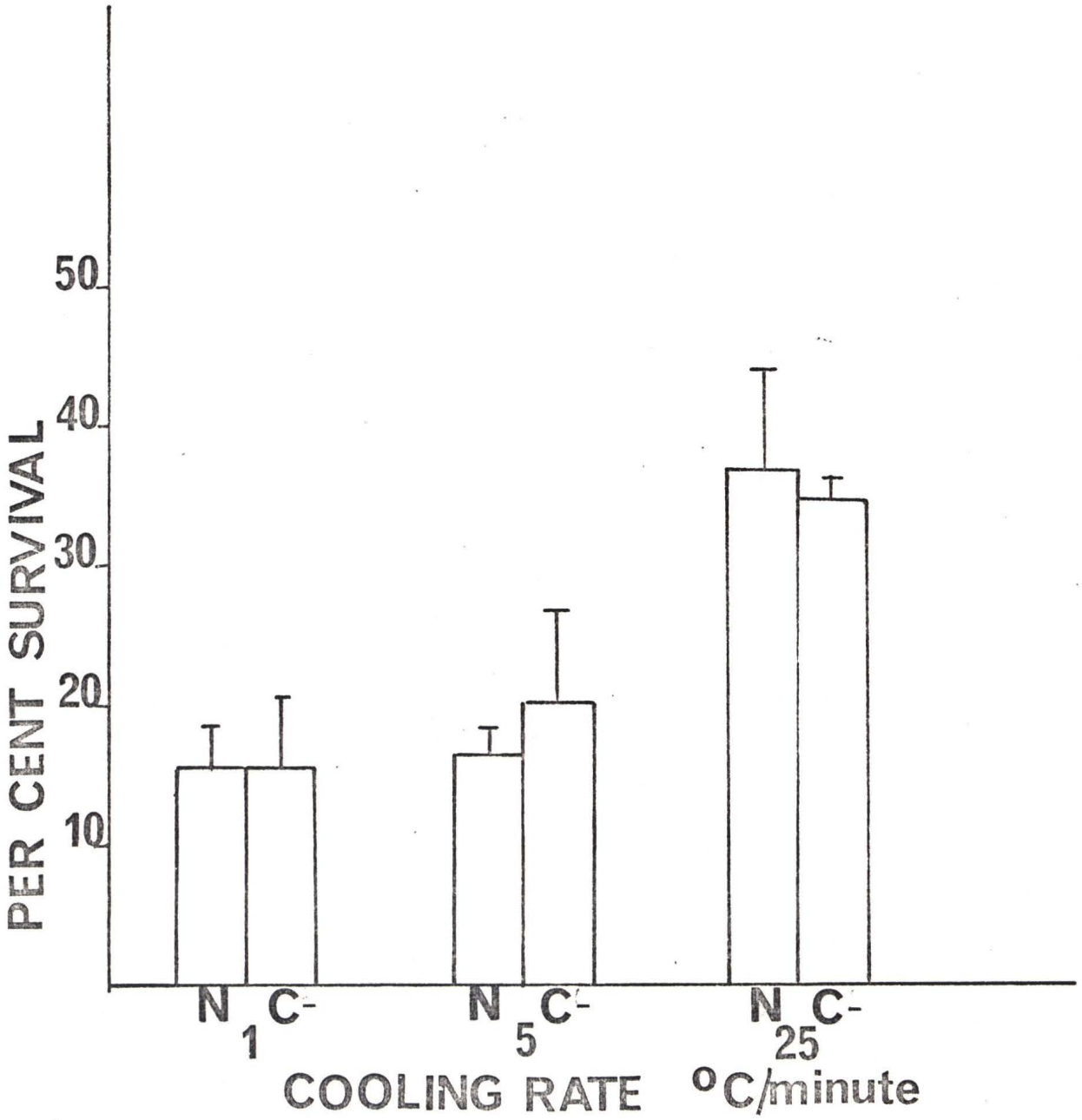
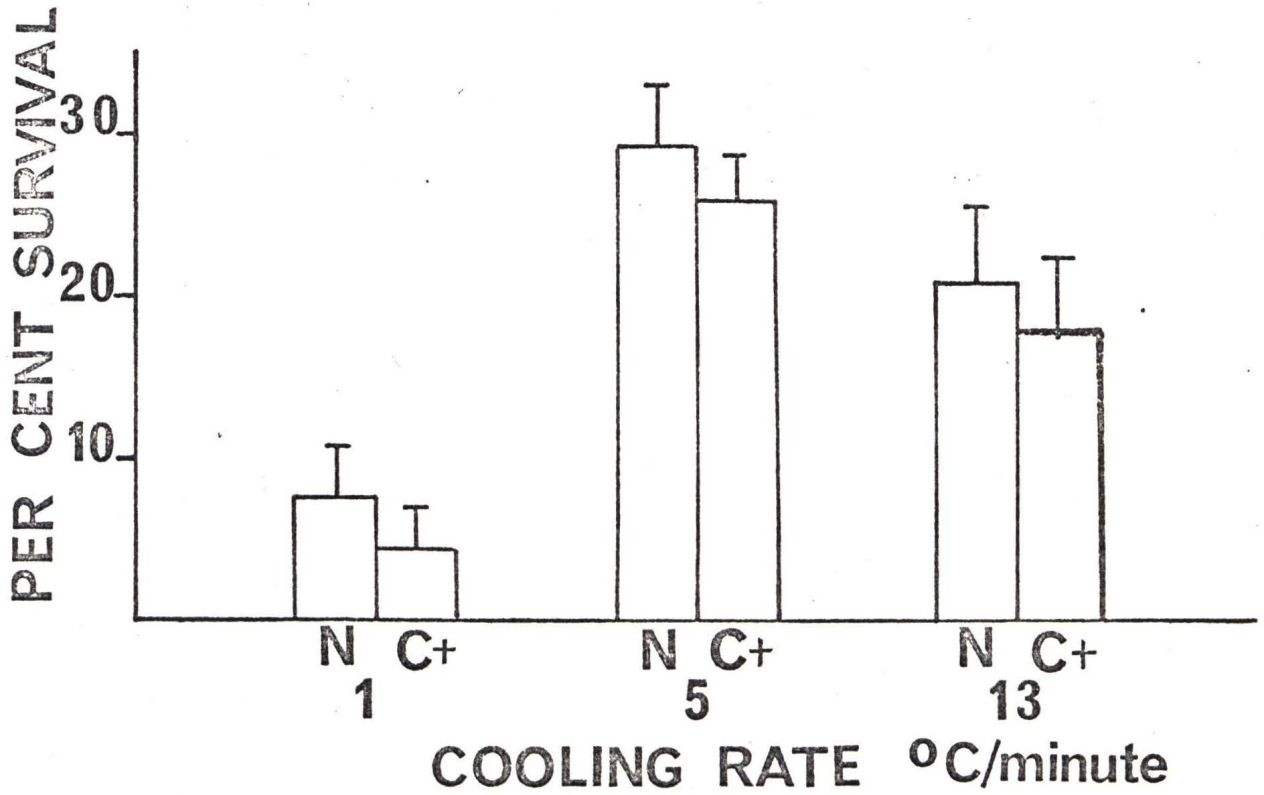

PER CENT SURVIVAL
50
40
30
20
10
N
C-
1
N
C-
5
N
C-
25
COOLING RATE °C/minute

Figure 23. Freeze/thaw survival of cells grown for 24 hours in normal medium or partially depleted medium (Batch 8). Cells were frozen in serum free medium plus 2.5% DMSO. Bar represents one standard deviation.

N Control

C^+ Cells in partially depleted medium. Cholesterol plus.


PER CENT SURVIVAL
30
20
10
N C+
1
N C+
5
N C+
13
COOLING RATE °C/minute

time spent above 0°C was 25 minutes during the slowest cool compared to 5 minutes at 5°C/minute and 2 minutes at 13°C/minute. Therefore, the least amount of freeze/thaw survival deviation between cell types frozen in normal medium with 2.5% DMSO should be manifested at the slowest cooling rate. This was not the case Figure 20. This was also confirmed when freeze/thaw survival of cholesterol plus cells and normal cells were compared at 5°C/minute and 13°C/minute in AMS 8 medium Figure 21. It would seem reasonable to conclude that serum cryoprotection occurred independently of cell changes during pre-incubation.

It would seem most reasonable to conclude from these data that the correlation between membrane cholesterol and freeze/thaw recovery was found only at slow cooling rates. This was the region of the cooling rate/survival curve where Frimm (1976) was able to achieve a change in the optimum cooling rate. In any case it was unlikely that survival at rates exceeding 5°C/minute was very dependent upon cholesterol supplementation with the exception of 25°C. If the membrane cholesterol *versus* freeze/thaw survival correlation were pertinent it should be manifested at all the cooling rates studied. These data would suggest that perhaps cell damage was quite different at different cooling rates. This would explain the irregularities in the cooling rate/survival curves found in other organisms (Table I).

There are several other reasons to be interested in

this correlation. Membrane cholesterol increases were shown to be correlated with osmotic shock and decreased permeability in many cell types and liposomes Jain, (1975). Decreased permeability by retarding dehydration should be correlated with increased freeze/thaw recovery. Cells would succumb to solution effects less readily. If osmotic shock were important at slow cooling rates, decreased survival should result. Appendix II demonstrated that AMS8 (Cholesterol plus) cells were very susceptible to osmotic shock. It would therefore, seem reasonable to conclude that there was a correlation between resistence to osmotic shock and freeze/thaw survival.

Darin-Bennet (1977) found that thermal shock in spermatozoa was correlated with increased membrane cholesterol. Raccah (1975) found that in mycoplasma increased membrane cholesterol was correlated with decreased freeze/thaw viability as confirmed here. These data support previous data suggesting little relationship between thermal shock and freeze/thaw Morris (1975).

Cholesterol has been reported to be found on the plasma membrane. Smaller amounts were found in lysosomes Elias (1978). These data would tend to implicate the plasma membrane as a target for damage at slow cooling rates. In erythrocytes and ascites tumor cells cholesterol depletion was correlated with increased lectin agglutination Alderson

(1978). Freeze/thaw viability may be related to membrane glycoprotein. This would account for a change in the optimum cooling rate in lymphocytes as reported by Knight (1972).

Lipid depletion of cells incubated in the lipid free medium was not pronounced presumably because the *de novo* cholesterol synthesis was not inhibited. Further support for the cholesterol correlation might be provided by cooling cholesterol mutants Chang (1977). Jain (1975) reported that lymphocytes had twice as much membrane cholesterol as lymphoma cells which might constitute another test. To distinguish between lysosomes and the plasma membrane as a freeze/thaw target, DMSO mutants might be invaluable.

The next section provides further support that cell viability was peculiar to the cooling rate used.

Fatty Acid Supplementation

Linoleic acid was added to cells in such a way as to maximize the probability of membrane incorporation thus altering the unsaturated/saturated fatty acid ratio. In these experiments cells were incubated in linoleate plus or minus avidin in lipid free medium for 24 hours before cooling at various rates in normal medium supplemented with 2.5% DMSO. Figure 24 demonstrated two optima for all cell treatments.These optima occurred at the same rates with the exception of linoleate plus avidin. At 1°C/minute, pre-incubation with linoleate plus avidin, yielded significantly different results from other treatments. In one experiment survival at 1°C/minute was very high in the control Figure 25. However, freeze/thaw survival after linoleate plus avidin treatment was similarly higher and significantly different at the 0.1% probability level. Only at 13°C/minute did freeze/thaw results parallel those obtained for the cell/cell adhesion test. Results here were not significantly different from the control.

When cells were frozen in lipid free medium plus 2.5% DMSO there were significant differences between LA and normal treatment at 1°C/minute Figure 27. At cooling rate 1°C/minute freeze/thaw recovery after LA treatment was significantly different from that of cholesterol minus cells at the 10% probability level. At 5°C/minute recovery after LA treatment was significantly different from normal treatment

Figure 24. Freeze/thaw survival in normal medium plus 2.5% DMSO of cells grown in normal, linoleate, linoleate plus avidin, or lipid free medium for 24 hours prior to freezing. Bar represents one standard deviation.

N Control

LA Cells in linoleate plus avidin in lipid free medium.

L Cells in linoleate in lipid free medium.

R Cells in lipid free medium.

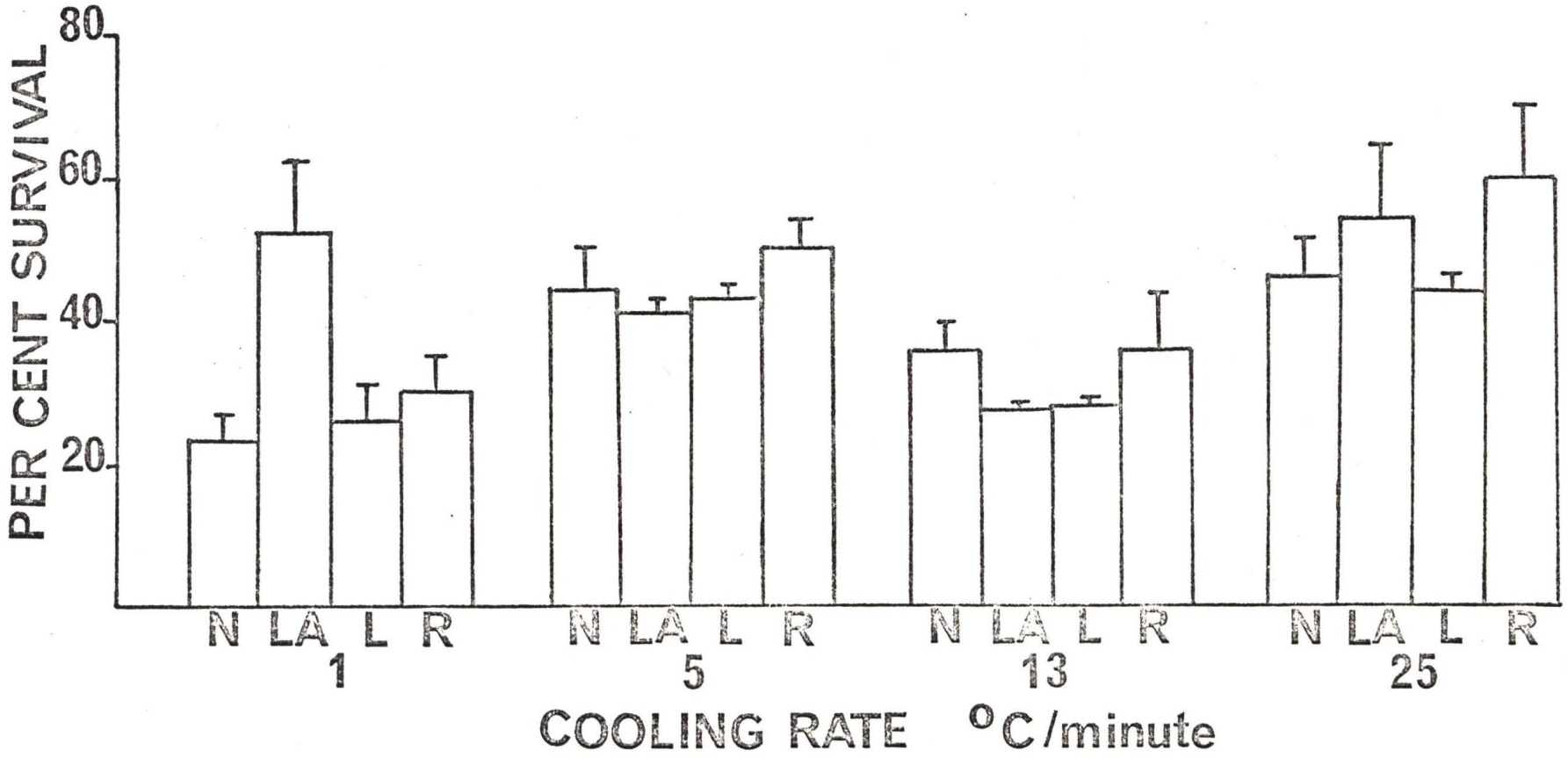

PER CENT SURVIVAL
80
60
40
20
N LA L R
1
N LA L R
5
N LA L R
13
N LA L R
25
COOLING RATE °C/minute

Figure 25. Freeze/thaw survival at 1°C/minute in normal medium plus 2.5% DMSO of cells grown in normal, linoleate, linoleate plus avidin, or lipid free medium for 24 hours prior to freezing. Bar represents one standard deviation.

N Control

LA Cells in linoleate plus avidin in lipid free medium.

L Cells in linoleate in lipid free medium

R Cells in lipid free medium.

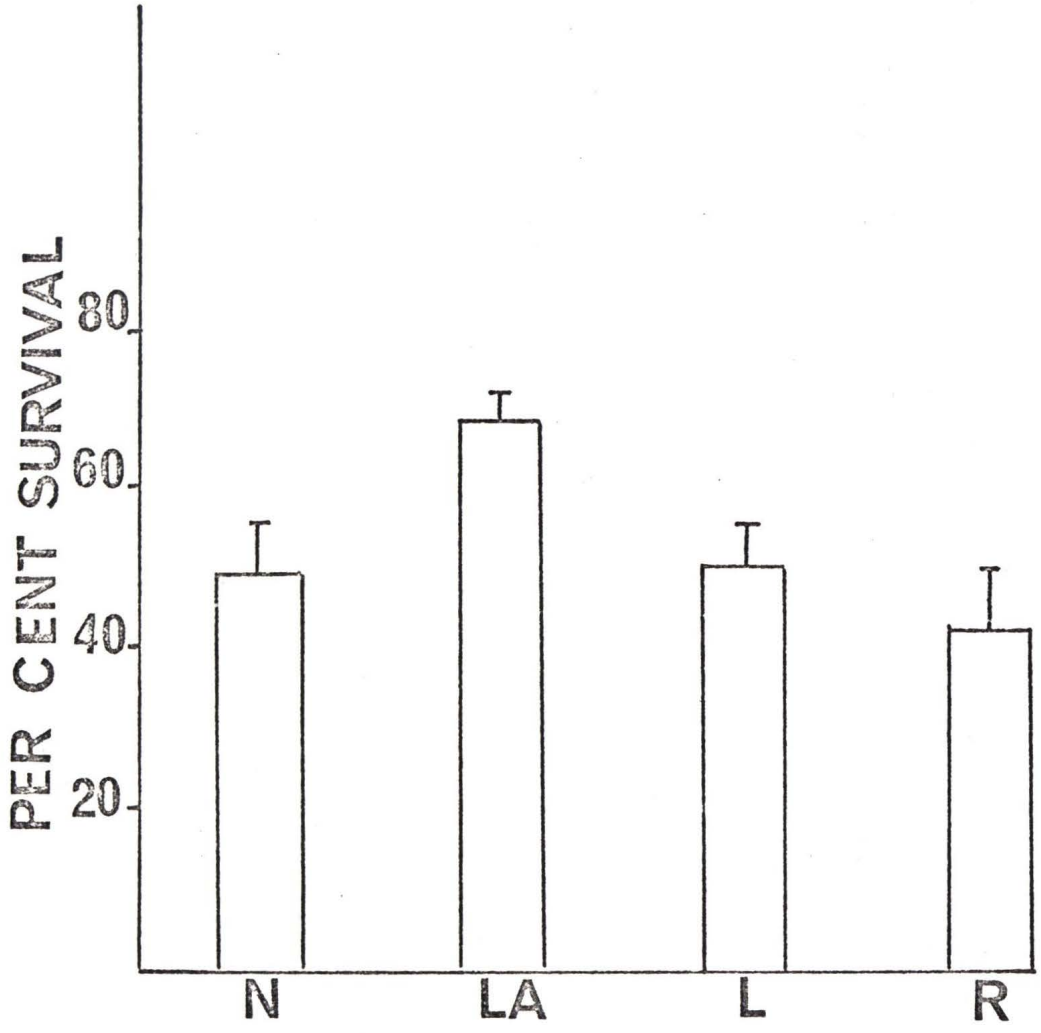

PER CENT SURVIVAL
80
60
40
20
N
LA
L
R

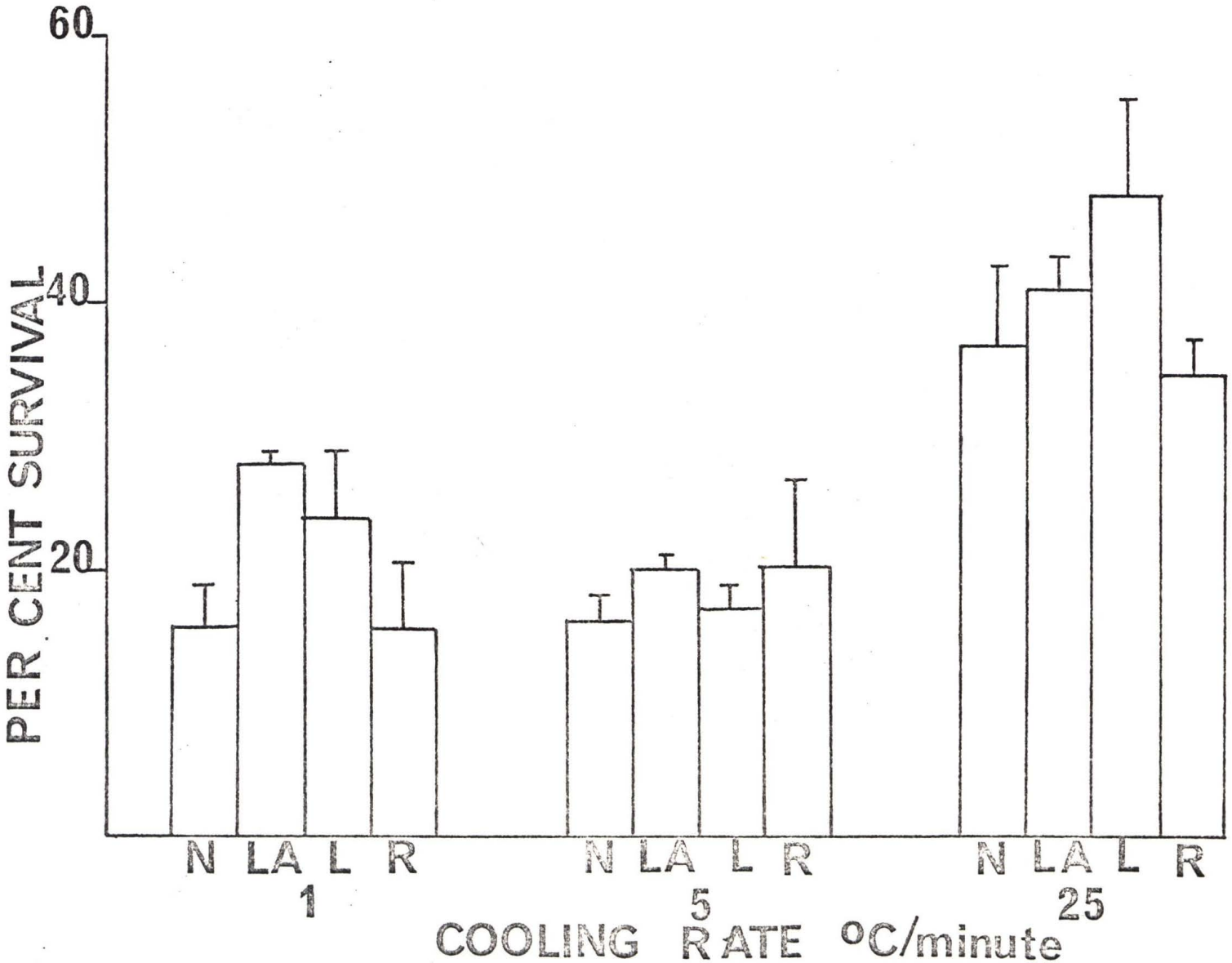
Figure 26. Freeze/thaw survival in lipid free medium plus 2.5% DMSO. Cells were grown in normal, linoleate, linoleate plus avidin, or lipid free medium prior to cooling. Bar represents one standard deviation.

N Control

LA Cells in linoleate plus avidin in lipid free medium.

L Cells in linoleate in lipid free medium

R Cells in lipid free medium.


60
40
20
PER CENT SURVIVAL
N LA L R
N LA L R
N LA L R
1
5
25
COOLING RATE °C/minute

at 5% probability level, and from linoleate treatment at 10% level. Freeze/thaw recovery of LA treated cells was not significantly different from that of the cholesterol minus (lipid free) cells. At 25°C/minute cell treatments gave similar survival. The shift in significance might be construed as evidence that lipid depleted medium exerted a traditional pattern of cryoprotection. While CHO freeze/thaw recovery after pretreating with linoleate and avidin was increased when cooling at 1°C/minute there was no evidence that a shift in the optimum cooling rate had occurred although survival was decreased at 13°C/minute. Changes in optimum cooling rates with CHO cells have been previously reported in this cooling rate range (0.3°C/minute to 3°C/minute) Frimm (1976).

Cells were also grown for 24 hours in lipid free medium supplemented with palmitic acid (2×10^{-5}M) plus or minus avidin prior to cooling and thawing at various rates Figure 27. At 1°C/minute normal cells yielded significantly lower freeze/thaw survival from any of the other treatments. However, palmitic acid treatments did not differ significantly from treatment with lipid free medium suggesting that increased survival might be attributed to incubation in lipid free medium rather than fatty acid supplementation. Survival after palmitic acid supplementation, at other cooling rates was not significantly different from other treatments.

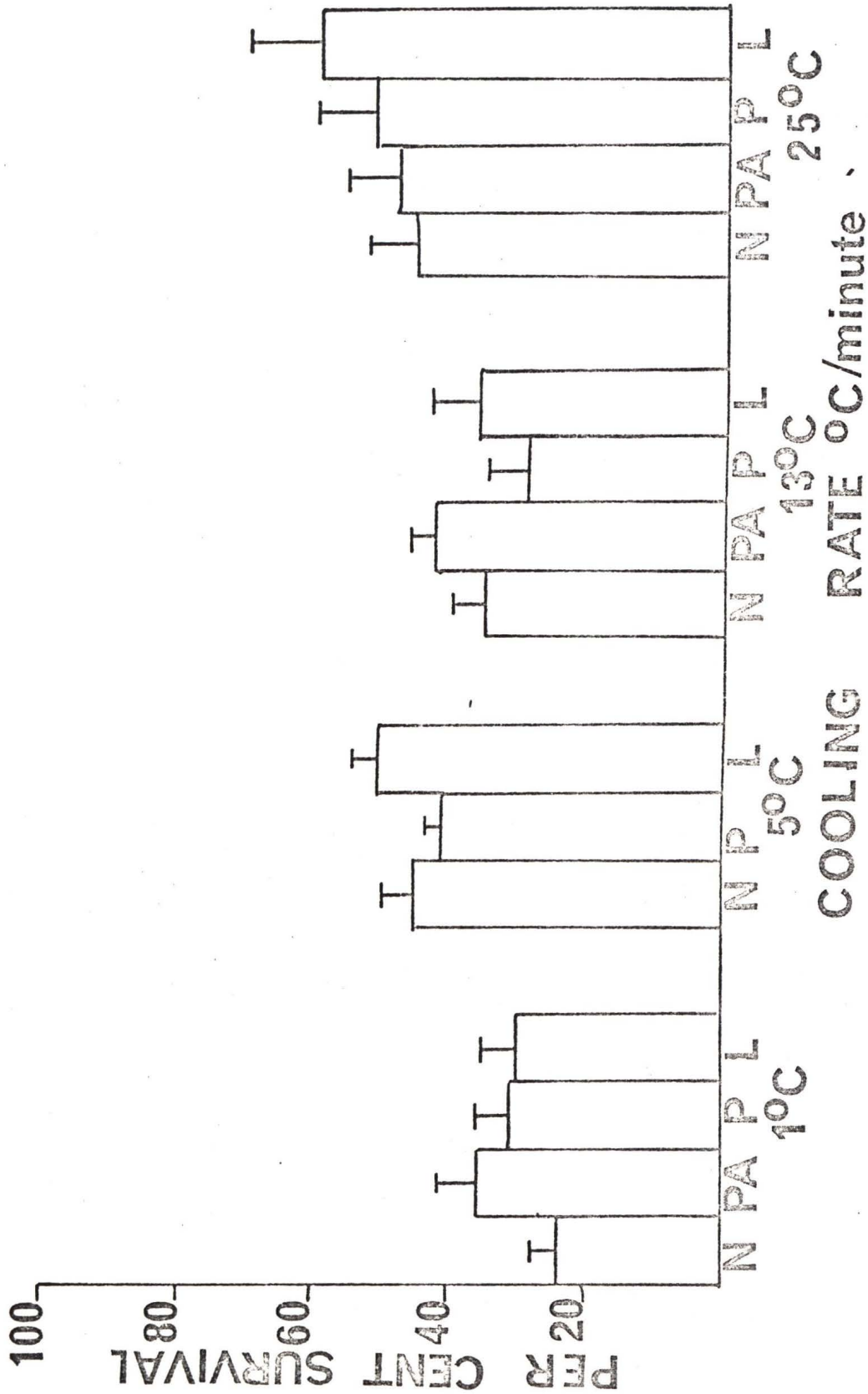
Figure 27. Freeze/thaw survival in normal medium plus 2.5% DMSO. Cells were grown in normal, palmitic acid, palmitic acid and avidin, or lipid free medium for 24 hours prior to cooling. Bar represents one standard deviation.

N Control

PA Cells in palmitic acid plus avidin in lipid free medium

P Cells in palmitic acid in lipid free medium.

L Cells in lipid free medium.


PER CENT SURVIVAL
100
80
60
40
20
N PA P L
1°C
N P L
5°C
N PA P L
13°C
N PA P L
25°C
COOLING RATE °C/minute

When freeze/thaw survival after linoleate treatment was compared to palmitic acid recovery there were no significant differences between LA, PA, L and P treatments at 5°C/minute and 26°C/minute Figure 28. Controls from previously performed experiments from which these data were collected were not significantly different from each other.

Only at 1°C/minute and 13°C/minute were there any significantly different results. LA cells gave significantly higher survival from other treatments at 1°C/minute. These experiments were compiled from three samples per cooling rate with at least two replicates/sample. All cells were treated with fatty acid supplementation in lipid free medium and frozen in normal medium with 2.5% DMSO. These data imply a shift in the optimum cooling rate has occurred with respect to LA and PA treatments since at 1°C/minute survival was higher with LA treatment than with PA treatment. At 13°C/minute the reverse was true. At 13°C/minute, PA and LA treatments had freeze/thaw survival which was not significantly different from lipid free control. This would dispute the interpretation of these data as a shift in the optimum cooling velocity. It would seem reasonable to conclude that although survival was altered at very slow cooling rates no such change was observed at faster one. No change in the optimum cooling rate was observed as assayed by freeze/thaw survival in the 1°C/minute to 25°C/minute range.

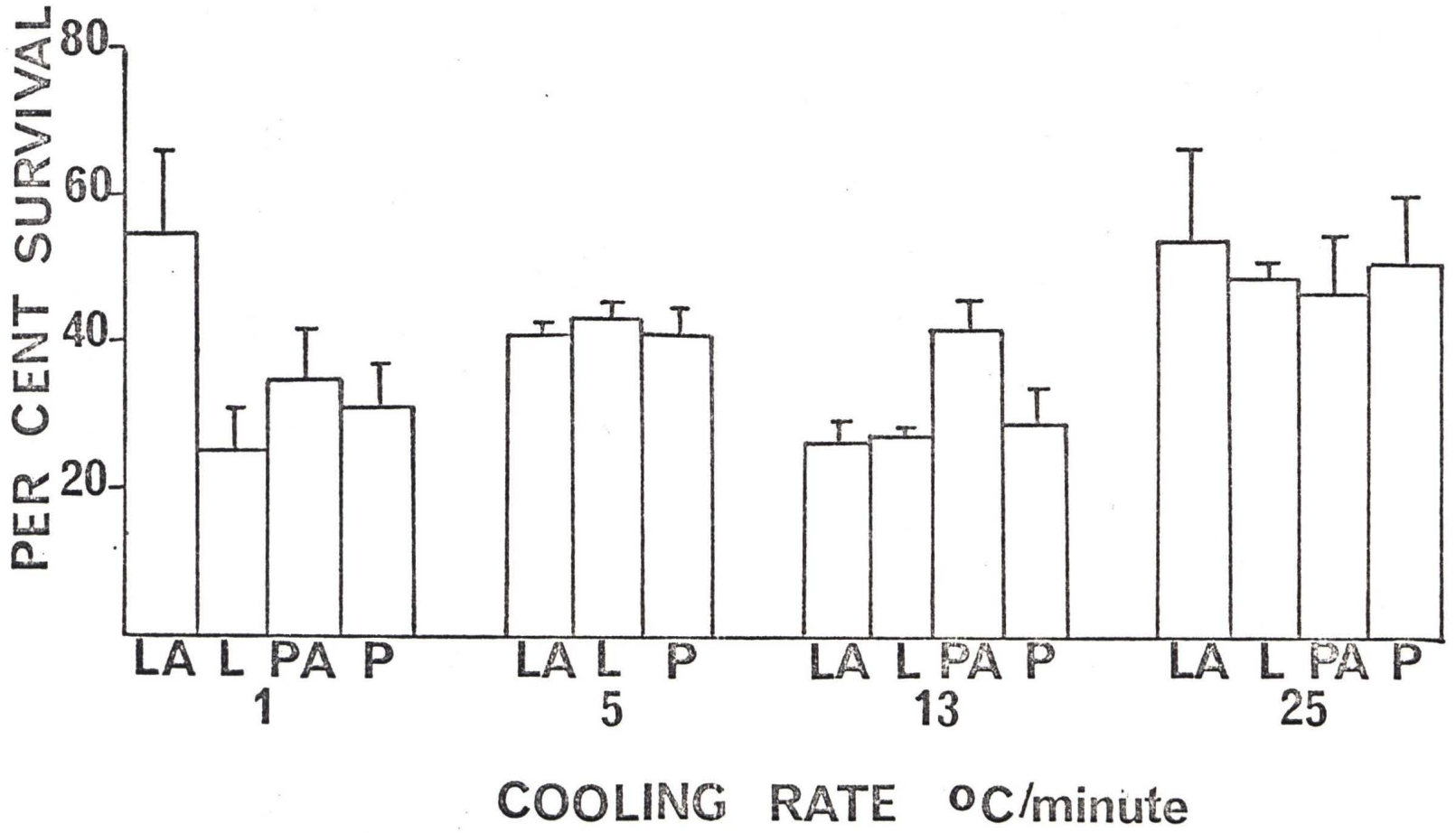
Figure 28. Freeze/thaw survival in normal medium plus 2.5% DMSO. Cells were grown in palmitic acid, palmitic acid plus avidin, linoleate, or linoleate plus avidin in lipid free medium prior to cooling. Bar represents one standard deviation.

PA Cells grown in palmitic acid plus avidin

P Cells grown in palmitic acid

LA Cells grown in linoleate plus avidin

L Cells grown in linoleate


PER CENT SURVIVAL
80
60
40
20
LA L PA P
1
LA L P
5
LA L PA P
13
LA L PA P
25
COOLING RATE °C/minute

The last effect to be assayed was the avidin one. If freeze/thaw viability at very slow cooling rates were due to avidin, then growing cells in medium supplemented with avidin should yield consistently higher than expected recovery. If fatty acid supplementation were important, then linoleate plus avidin pre-treatment should yield the highest freeze/thaw survival. If due to a metabolic change which may have terminated fatty acid synthesis, then avidin effects should be most pronounced with cells incubated in lipid free medium. Cells were therefore grown in linoleate plus avidin in lipid free medium, palmitic plus avidin in lipid free medium, lipid free medium plus avidin, or normal medium plus avidin for 24 hours before cooling at 1°C/minute and 26°C/minute Figure 29 and 30. Palmitic plus avidin was used as a control since as previously discussed, avidin had no effect on survival at slow cooling rates. All results at 1°C/minute with the exception of PA treatment were from significantly different from respective controls. Treatment with avidin in lipid free, normal and lipid free medium plus linoleate were not significantly different from each other at 1°C/minute.

At 25°C/minute, Figure 30, results were not significantly different from one another. To determine if the effect was reversible, cells were treated as above then incubated in normal medium for various times before cooling at 1°C/minute. If the effect were metabolic, one might expect immediate reversibility as suggested by Horowitz (1974).

Figure 29. Freeze/thaw survival in normal medium plus 2.5% DMSO. Cells were grown in normal, linoleate, palmitic acid, or lipid free medium plus or minus avidin and frozen at 1°C/minute after 24 hours. Bar represents one standard deviation.

N Control

L Cells grown in linoleate in lipid free medium

P Cells grown in palmitic acid in lipid free medium

R Cells in lipid free medium.

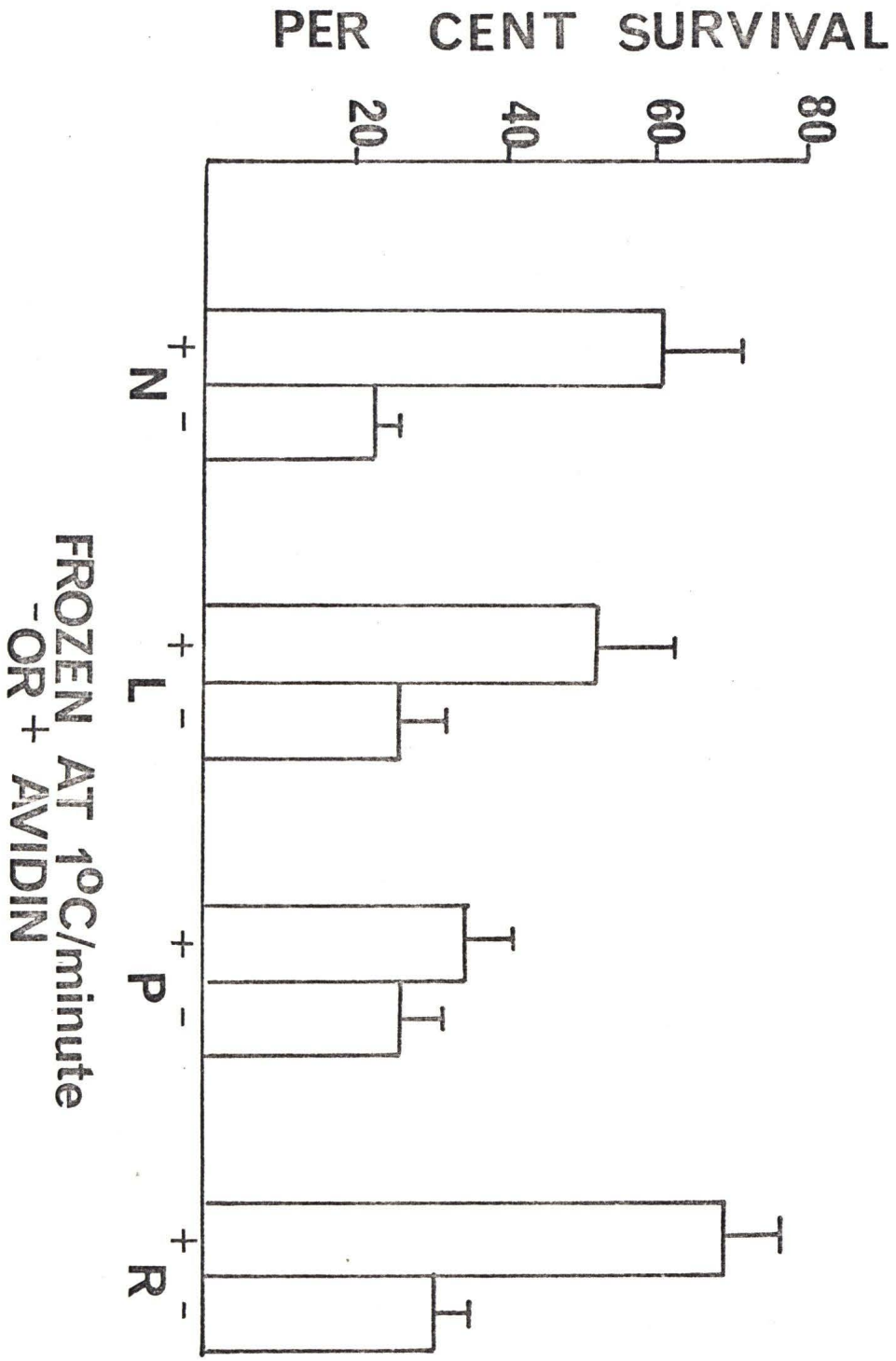

PER CENT SURVIVAL
20
40
60
80
N
L
P
R
+
-
FROZEN AT 1°C/minute
-OR+ AVIDIN

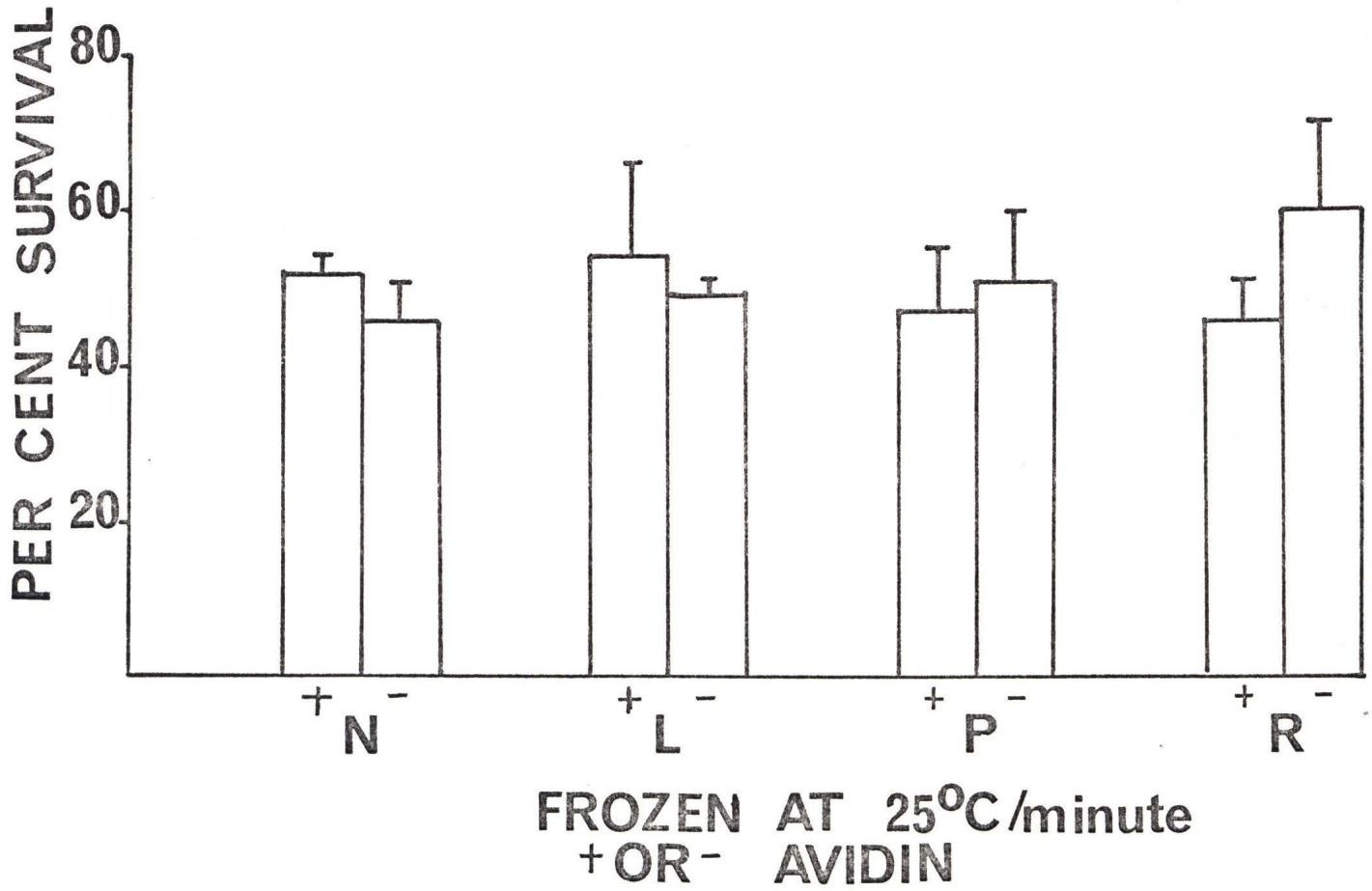
Figure 30. Freeze/thaw survival in normal medium plus 2.5% DMSO. Cells were grown in normal, linoleate, palmitic acid, or lipid free medium plus or minus avidin and frozen at 25°C/minute after 24 hours. Bar represents on standard deviation.

N Control

L Cells grown in linoleate in lipid free medium

P Cells grown in palmitic acid in lipid free medium

R Cells in lipid free medium


PER CENT SURVIVAL
80
60
40
20
+ N −
+ L −
+ P −
+ R −
FROZEN AT 25°C/minute
+OR− AVIDIN

After 4 hours in medium without avidin, normal (NA) cells demonstrated a tendancy to revert to normal freeze/thaw survival though survival was still twice that found regularly. Linoleate data did not indicate any reversal. Only in lipid free medium was there any substantial change in survival. PA cells gave survival expected for normal cells Figure 32.

Changes in membrane fluidity have not been reported to alter the number of receptors on the membrane, although the degree of binding especially at lower temperatures decreased as assayed by lectin agglutinability and virus absorption Horowitz (1974); Li (1975); Mahoney (1977). It is possible that freeze/thaw survival was closely linked to endocytosis which has been shown to be least pronounced in cells with a high saturated/unsaturated fatty acid ratio Mahoney (1977). It may be that incubating normal cells in medium with avidin might accelerate the degree of polyenoic membrane fatty acid incorporation which would increase the fluidity. Alderson (1978) reported that lectin agglutinability in cholesterol depleted cells at room temperature was more pronounced than in control. It may be that freeze/thaw survival is a function of membrane fluidity. Knight (1972) found that pretreatment with mitogen was successful in altering freeze/thaw survival at slow cooling rates. In any case further experiments must be performed to ascertain the nature of the avidin effect.

Figure 31. Freeze/thaw survival in normal medium plus 2.5% DMSO when samples were seeded and thawed slowly. Samples were cooled at 1°C/minute after incubating in normal, linoleate, palmitic, or lipid free medium supplemented with avidin. Bar represents one standard deviation.

N Control

L Linoleate plus avidin in lipid free medium

P Palmitic acid plus avidin in lipid free medium

R Lipid free medium plus avidin

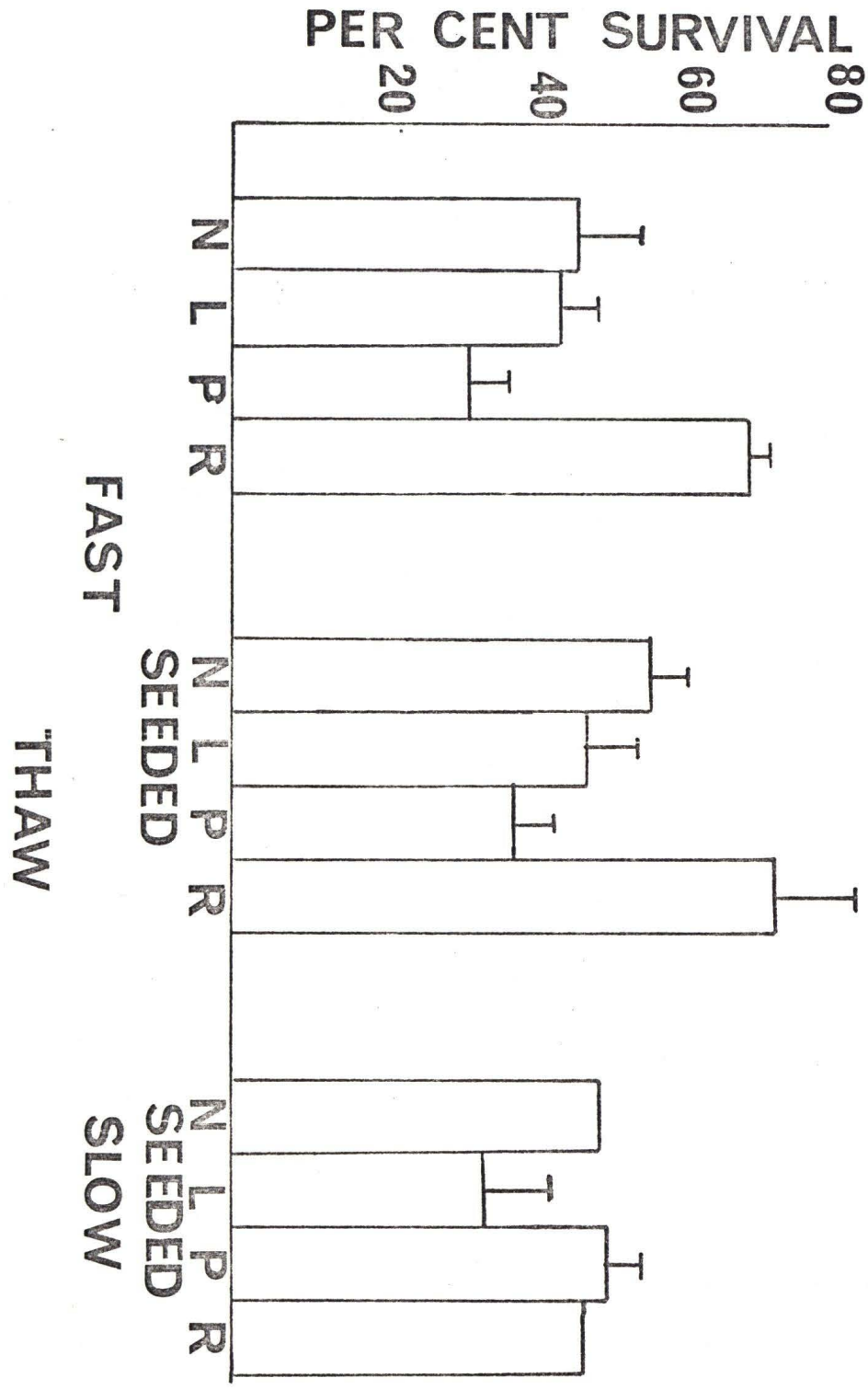

PER CENT SURVIVAL
20
40
60
80
FAST THAW
N
L
P
R
SEEDED
N
L
P
R
SLOW SEEDED
N
L
P
R

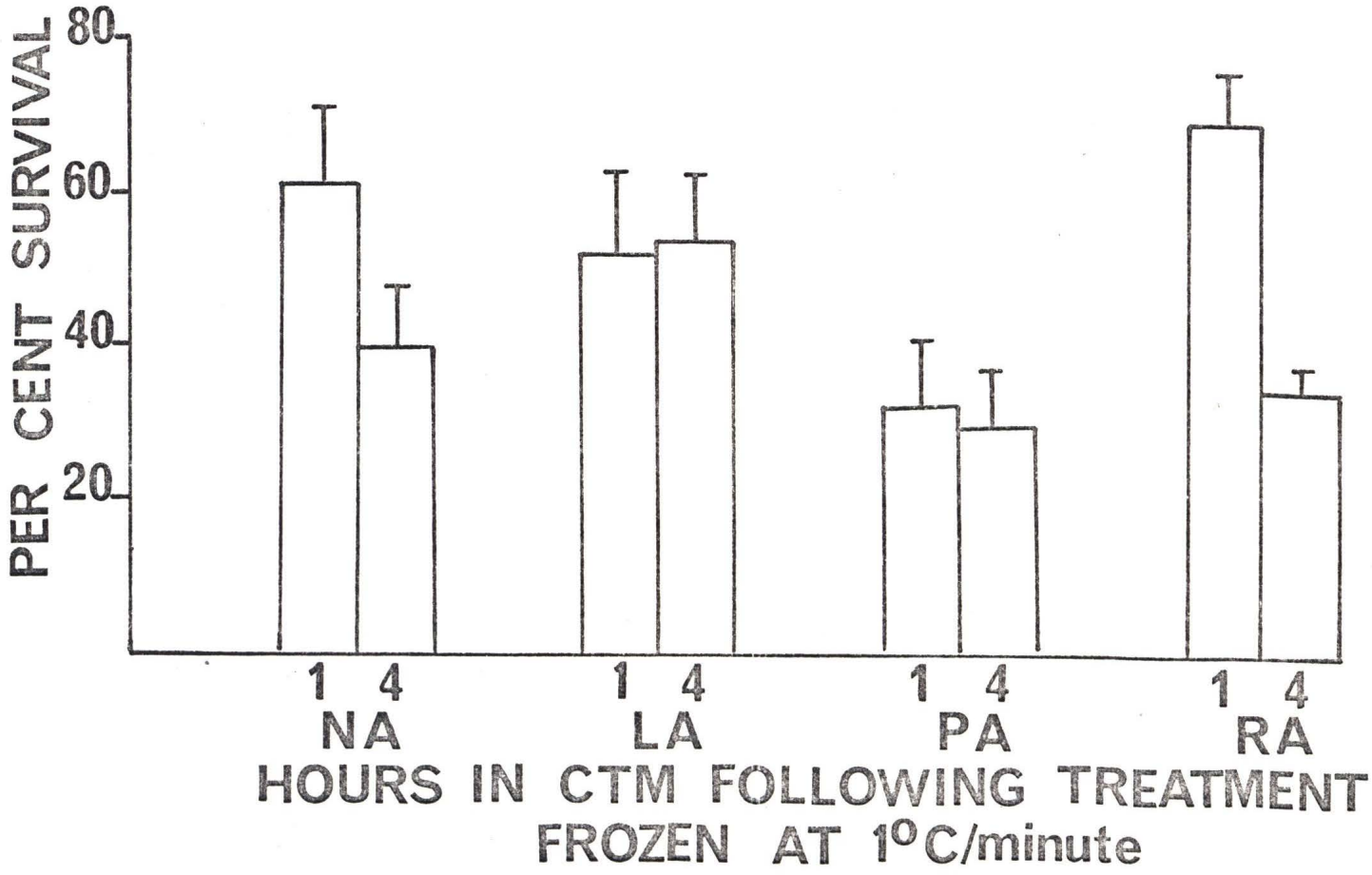
Figure 32. Freeze/thaw survival in normal medium plus 2.5% DMSO after incubating cells in normal, linoleate, palmitic acid, or lipid free medium plus avidin and changing to normal medium for 1 and 4 hours prior to cooling. Bar represents one standard deviation.

NA Control

LA Linoleate plus avidin in lipid free medium

PA Palmitic acid plus avidin in lipid free medium

RA Lipid free medium plus avidin


PER CENT SURVIVAL
80
60
40
20
1 4
NA
1 4
LA
1 4
PA
1 4
RA
HOURS IN CTM FOLLOWING TREATMENT
FROZEN AT 1°C/minute

In all these experiments, the only cooling rate where survival was consistently altered was at 1°C/minute. Results from cooling rates of 25°C/minute were in agreement with those previously reported by Mazur (1972) and Connor (1973). The optimum cooling rate in 2.5% DMSO has been reported to be about 50°C/minute. This presumably was where intracellular ice formed. It would seem reasonable to conclude that each of these treatments reported were not influenced by this parameter since survival at 25°C/minute was always similar with the exception of Cholesterol minus cell freeze/thaw recovery.

Results from lipid depleted medium implied that this system was sensitive to changes in the optimum cooling rate yet no treatment reported here was successful in effecting such a change. It would seem reasonable to conclude that there might be an additional parameter to be assayed or that survival was peculiar to the cooling rate used. The freeze/thaw survival changes reported here occurred in the same cooling rate range used by (1976). This would suggest that changes in optimum cooling rate may be confined to even slower rates than were used here.

CONCLUSIONS

This thesis provides support for the corrollory between egg yolk and fetal calf serum protection. Pace (1975) and Morris (1975) implicated the egg yolklipid constituents in cryoprotection. Two different lipid-depleting techniques substantiated the postulate that lipids were the important cryoprotective agents in fetal calf serum as well.

It is unlikely that fetal calf serum protected against thermal shock. Raccah (1975) reported that decreased membrane cholesterol was correlated with increased freeze/thaw survival while Darin-Bennet (1977) found that increased cholesterol on spermatozoa plasma membranes protected against thermal shock. In this thesis decreased membrane cholesterol was correlated with increased freeze/thaw survival. These data therefore confirm Morris' (1975) suggestion that there was a lack of correlation between thermal shock and freezing and thawing.

The most interesting data in this thesis were the avidin and cholesterol effects. Both were confined to slow cooling rates of less than 5°C/minute. At faster rates of 20°C/minute and 13°C/minute freeze/thaw survival was not significantly different than control. That this system was sensitive to changes in the optimum cooling rate was substantiated by the data on fetal calf serum protection. Depleting serum of lipids was correlated with a change in

the optimum cooling rate to higher velocities.

Furthermore freeze/thaw survival profile reported here repeated that of Mazur (1972) and Connor (1973) implying that these data were not due to experimental error. Intracellular ice was postulated to form at 50°C/minute. The rates studied here were far slower. This would suggest that there was indeed an additional unaccounted for variable.

According to the models reviewed, dilution shock, osmotic shock and thermal shock should become increasingly detrimental as exposure to hypertonic solutions was increased. This does not explain the double optima observed in these and other reported experiments. The easiest explanation for these results is to assume that survival versus cooling rate curve was biologically determined, and that biological damage varied with the cooling rate.

The cholesterol datum gives some insight into the kind of damage. Cholesterol increases predisposed the cells to osmotic shock as reported by this thesis. This would imply that this insult was of importance at slow cooling rates. Cholesterol in various cell types was confined to the plasma membrane and lysosomes Elis (1978). This would implicate both of these membranes in freeze/thaw recovery at slow cooling rates if this correlation were meaningful.

The avidin effect was not investigated in this thesis and remains to elucidated. Twelve different experiments attest to its repeatability.

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APPENDIX I

Thin layer chromatography on various batches of AMS serum revealed the inadequacy of AMS treatment in depleting serum. The same panorama was revealed as in normal serum. The ethanol/ether treatment was successful in eradicating all lipids.

Individual lipid calculations were performed barring glyceride calculations. The only lipid consistently depleted was the phospholipids. The cholesterol/phospholipid was consistently higher than in normal serum.

Electrophoretograms revealed no specific selection of a protein class.

Figure 33. Electrophoretograms of protein in lipid free medium, partially lipid depleted medium, and normal medium.

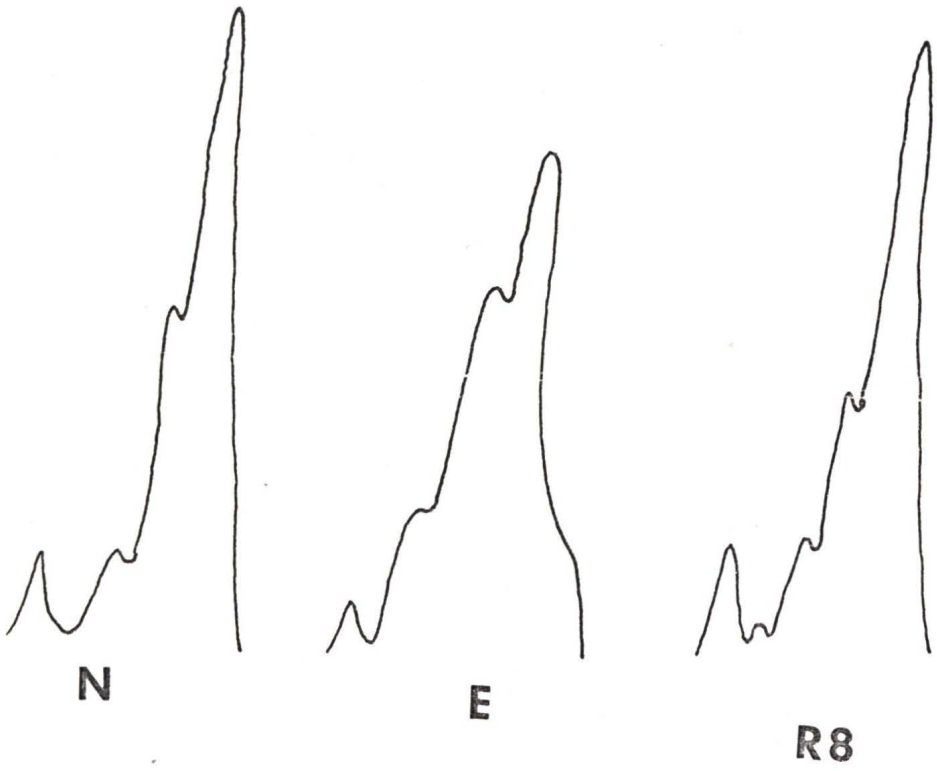

N
E
R8

Figure 34. Thin layer chromatography of normal, partially depleted, and lipid free medium.

O Oleate control

P Palmitate control

C Cholesterol control

D Diglyceride control

M Monoglyceride control

T Triglyceride control

R Ammonium sulfate medium

N Normal medium

E Ethanol/ether medium

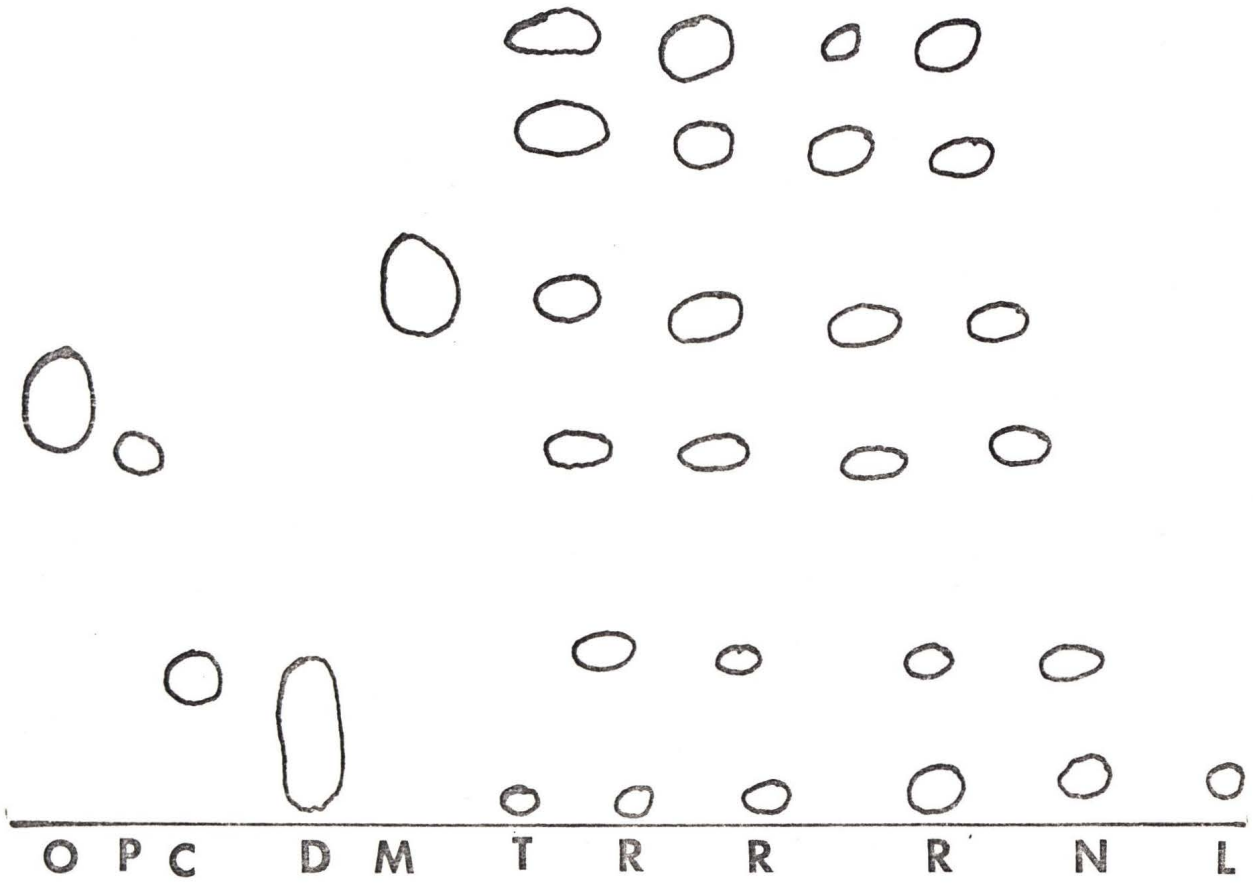

O P C D M T R R R N L

TABLE V

Calculation of Phospholipid, Fatty Acid, and Total Cholesterol per ml of CTM

Medium	Phospholipid	Fatty acid	C	C/P
Normal	0.054	0.52	0.0295	0.55
S.D.	0.0036	0.09	0.005	
AMS 6	0.014			
AMS 7	0.014			
AMS 8	0.0135	0.27	0.026	1.90
AMS 9	0.023			
AMS 10	0.014	0.52	0.0156	1.12
AMS 11		0.73	0.048	3.40
Ether	0	0	0	0

Organic Phosphate µmoles

Fatty acid µmoles

Cholesterol (C) µ grams

C/P Cholesterol/phospholipid

Appendix II

TABLE VI

Cholesterol Determinations on Cells Incubated in Different Media

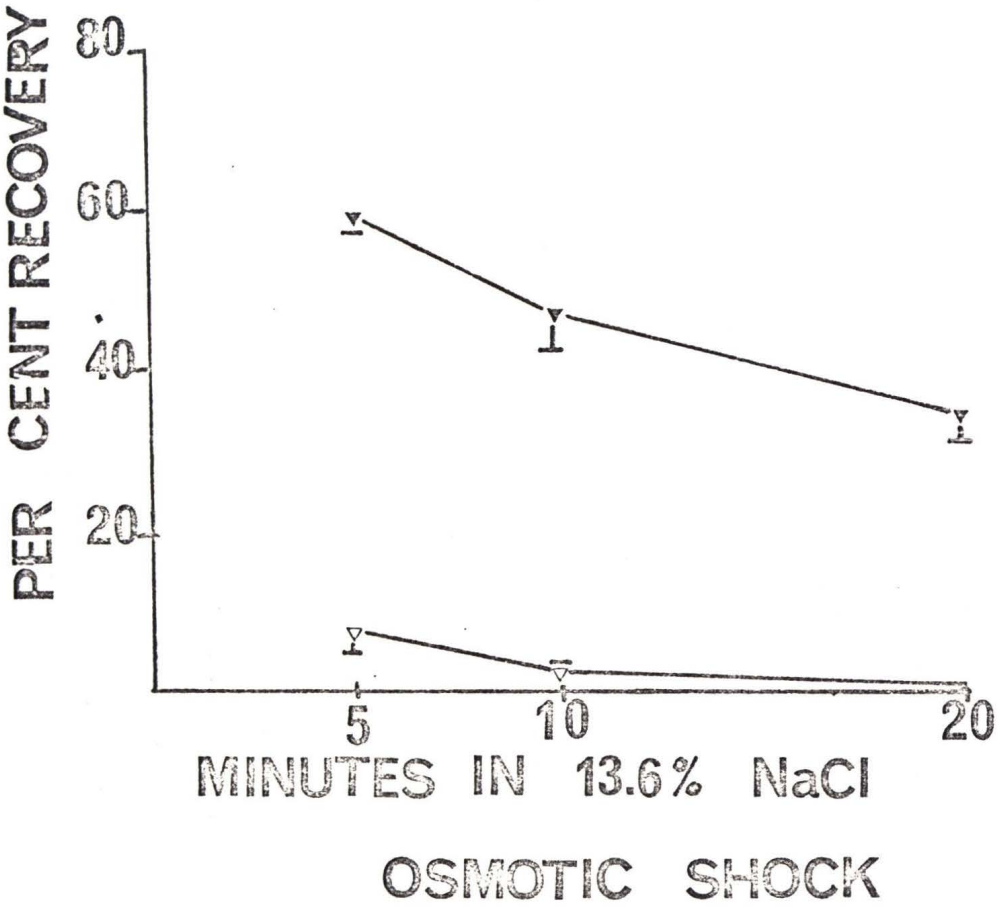
Medium	Cholesterol Esterase % Survival	Cholesterol %
Control cells	29.1	31.5
AMS 8 cells	12.4	50.5
Ethanol/Ether cells	79.5	27.4

Jain (1975) suggested that sterol addition contributed to osmotic fragility as was demonstrated below with AMS 8 medium.

Figure 35. Osmotic fragility of cells incubated in normal and partially depleted medium for 24 hours prior to incubation in 13.6% NaCl solution in CTM without serum.

▼ Control

▽ Cells in partially depleted lipid medium Batch 8


PER CENT RECOVERY
80
60
40
20
5
10
20
MINUTES IN 13.6% NaCl
OSMOTIC SHOCK

VITA

Surname: GOSHKO Given Names: SUSANNE LYNN

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Title of Thesis/Dissertation

GROWTH CONDITIONS AND FREEZE/THAW SURVIVAL IN CHINESE HAMSTER OVARY CELLS

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