THE INFLUENCE OF LEAKAGE MATERIALS IN FOUR FROZEN CELL SUPERNATANT FLUIDS ON THE RESTORATION OF ESCHERICHIA COLI (#11303) FROZEN CELLS

Thesis for the Degree of M. S.

MICHIGAN STATE UNIVERSITY

Thomas L. Roszman

1963

THE INFLUENCE OF LEAKAGE MATERIALS IN FOUR FROZEN CELL SUPERNATANT FLUIDS ON THE RESTORATION OF ESCHERICHIA COLI (#11303) FROZEN CELLS

By

Thomas L. Roszman

A THESIS

Submitted to

Michigan State University

in partial fulfillment of the requirements

for the degree of

MASTER OF SCIENCE

Department of Microbiology and Public Health

1963

ACKNOWLEDGEMENTS

The author wishes to express his sincere appreciation to Dr. F. R. Peabody for his guidance and support throughout this work.

Appreciation is also extended to Dr. W. L. Mallmann for his assistance and counsel.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

The effect of freezing on microorganisms has been the subject of a great many papers. The early reports were primarily concerned with time-temperature relationships and their effect on the per cent recoveries after freezing. Only recently have experiments been designed to elucidate the mechanism of freeze death and injury. As yet, no one mechanism proposed to account for death and injury, has gained wide acceptance. Indications are that the mode of action may be dependent on experimental conditions.

One of the more prominent theories to explain the action of freezing and thawing is that of metabolic damage. It has been shown that organisms, after being subjected to freezing and thawing, are injured metabolically. This has been found to be manifested in various ways such as: increased nutritional requirements, inability to grow on selective media, and loss of motility. The reason for this metabolic injury is not clear. Several investigators, such as Hartsell (1959, 1961), believe that it is caused by the loss of cellular constituents due to cell leakage. It is the purpose of this work to determine whether or not cell leakage,

loss of cellular material without lysis, is occurring with cells of Escherichia coli frozen in various suspending agents, and if this leakage material can affect the respiration rate of frozen E. coli.

LITERATURE REVIEW

I. General Aspects of the Effect of Freezing On Microorganisms

Microorganisms, when subjected to sub-zero temperatures, give a broad spectra of damage and death, dependent not only on the obvious experimental variants, but also on more subtle ones. One must not only be concerned with the species of organisms, time, temperature and rate of freezing, suspending agent, thawing time and temperature, but with the past history of the organism. The method of culturing the organisms is just as important as the time and temperature of freezing. Since each investigator has established his own system to study the effect of freezing, care must be taken in comparing the various experiments and drawing conclusions. To complicate matters, freezing seems to have a built-in inconsistency, which invariable effects each experimental trial (Campbell, 1943; Eaum, 1943).

Effect of sub-zero temperature. Macfayden and Rowland (1900 a) observed that a wide variety of microorganisms could survive freezing at -190 C for one week. They made no attempt to determine the number surviving after freezing. Smith and Swingle (1905), also using

Salmonella typhosa suspended in broth, they found a 99.5 % reduction in two hours at -17.8 C, and 99.3 % reduction for the same time of storage at -190 C. On the basis of this evidence, they concluded that reduction in viable cell number was as great at -17.8 C as at -190 C. Freezing at -185 C and using the colon bacillus as the test organism, Rivers (1927) found approximately the same percentage reduction. However, he employed several freezings and thawings, which are known to cause much greater destruction and damage (Smith and Swingle, 1905; Hilliard et al., 1915). Weiser and Osterud (1945), using E. coli suspended in 1.0 % peptone water, found that freezing at -195 C, and immediately thawing, gave a 55.1 % reduction.

Other temperature ranges have also been used to determine their effect on microorganisms. Bacteriophage have sustained no loss in titer, when frozen at -78 C Sanderson, 1925). Weiser and Osterud (1945) noted better survival with E. coli at -78 C, than at -5, -15, or -30 C.

Most investigators have found that temperatures above -30 C are more lethal to microorganisms than below this temperature. Hilliard et al. (1915), using E. coli and Bacillus subtilis, found that -15 C was more damaging than -2 C. However Haines (1938), also using E. coli, pointed out that storage at -2 C caused more

reduction in viable cell counts than storage at lower temperatures. Turner and Brayton (1939) found no loss of activity in various spirochetes and filterable viruses at -78 C, but storage at -10 or -20 C, was followed in the case of the spirochetes by loss of virulence and death.

More recently, Mazur (1940 a) has stated that Saccharomyces cerevisiae and Aspergillus flavus, frozen in distilled water, were drastically reduced in number between the temperature range of -10 and -30 C. In the case of Saccharomyces, this was not in agreement with Goetz and Goetz (1938). Although the experimental conditions were quite similar, they found that the greatest injury occurred in the vicinity of -50 C. They did, however suspend their organism for freezing, in Ringer's solution which would account for the discrepancy.

Effect of storage time. Not only is the temperature at which microorganisms frozen important, but also the length of storage. Prudden (1887) established that when bacteria were frozen, a large percentage exhibited an "immediate death," followed then by a more gradual storage death. This has proven to be one of the few instances in the literature of low temperature microbiology where general agreement was found.

Salmonella typhosa, suspended in saline and frozen at -190 C was found by Winchester and Murray (1936) to be reduced from $1x10^6$ to $1x10^4$ viable organisms in twenty-four hours. But no further drop was noted for samples examined daily for the next six days. Weiser and Osterud (1945) also found similar results using E. coli. The cells were suspended in 1.0 % peptone and frozen at -195 C. Plate counts were made on samples thawed immediately after five hours and ten hours of storage at -195 C. The percentage reductions were the same for all three samples, showing clearly that the greatest reduction occurs immediately after freezing. Reeves and Harrison (1957) again using E. coli but suspending in 4.6 M sodium chloride, observed that the greatest per cent reduction appeared in the first twenty-four hours of freezing at -9 and -22 C, followed by a more gradual decline.

The only extensive work done on the effect of storage time using a wide variety of microorganisms was done by Jones and Fabian (1952). Eighty cultures of bacteria representing eleven different genera were subjected to freezing at -17.8 C. Twenty-eight of these cultures were thermophiles, thirty were mesophiles, and seventeen were psychrophiles. Sampling was done before freezing and

after, at 4, 8, 12, 24, 48, 72, 96, 120 hours, and after six weeks. The sharpest drop in cell counts occurred during the first twenty-four hours, followed then by a more gradual reduction as storage time progressed.

Effect of cooling and warming rate. Little work has been done on the effect of cooling and warming rates and their relationship to the freezing and thawing of microorganisms. With only a few exceptions, have investigators reported the rates of cooling and warming. However, it is a well-known fact that solutions which are rapidly frozen, have different physical and thermodynamic characteristics, than solutions which are slowly frozen. It is entirely possible that these differences can be translated into the effect they would have on the microorganisms suspended in the solutions.

Mazur, in a series of four papers (Mazur et al., 1957 a, 1957 b; Mazur, 1960 a, 1960 b), has done the only extensive study on cooling and warming rates, and their effect on the freezing microorganisms. Their test organisms were Saccharomyces cerevisiae, Aspergillus flavus, and Pasteurella tularensis, suspended in a wide variety of menstra for freezing. Essentially, they found a slow cooling rate (1 C/min) favored microbial survival, while a rapid cooling rate (50 C/min) was detri-

mental. But when thawing was carried out, a rapid warming rate was better than a slow one. Rapid thawing also favored survival of spirochetes as opposed to slow thawing (Turner and Drayton, 1939).

Effect of suspending fluids. The menstra in which cells are suspended for freezing, plays a large part in the survival of the cells. In fact, the suspending fluid is probably more important than any other factor in the effect of freezing on microorganisms. Depending upon which suspending agent is used, per cent recoveries may range 1 to 99 %.

Various menstra have been used to suspend organisms for freezing. Freezing E. coli in milk, Keith (1913) found that little destruction took place. However, if the milk were diluted with water, the reduction became greater. Tap water proved to be the poorest menstrum used--only 1 % survival at -20 C for five days. Distilled water when used as a suspending agent, gave just the reverse results--about 95 % recovery (Clement, 1961; Harrison, 1956).

Cream containing 30 % butter fat, afforded good protection to E. coli and B. subtilis (Hilliard et al., 1915). This is not surprising since cream would act much like milk. More quantitative results were presented by Clement (1961), who froze E. coli in skim milk (12% Difco) at -78 C for two minutes. Eighty-six per cent

of the organisms could be recovered.

Many other suspending agents have provided good protection to organisms upon freezing. Beef-blood serum, 7.5 % glucose, 4.5 % glycerol, beef serum and 7.5% glucose gave high per cent recoveries, with E. coli frozen at -78 C for two minutes (Clement, 1961). Jones and Fabian (1952) obtained their best survival by freezing various organisms in vegetable extract and 40 % sucrose solutions. Postgate and Hunter (1961) also found that high molarities of glucose and sucrose, favor high survival rates.

Glycerol, which is a well-known stabilizer of other biological systems, also has the same effect on microorganisms. Gram positive and gram negative organisms suspended in Albimi brucella broth containing 15.0 % glycerol, survived well after five months at -10 C (Howard, 1956). The presence of 15 % glycerol, protected E. coli, Diplococcus pneumoniae, and Treponema pallidum from damage by freezing and thawing (Hollander and Nell, 1954).

Suspending of microorganisms in saline and buffer systems has also been reported. A comparison of saline and broth suspending media was made by Proom and Hemmons (1949). Six organisms were used. Freezing was carried out at -78 C. In all instances, recovery was four to eight

times greater in broth, with the exception of Staphylococcus aureus where 100 % survival was found in both saline and broth. Freezing E. coli in saline resulted in greater injury than those suspended in nutrient broth (Nakamura and Dawson, 1962). Organisms suspended for freezing in a high concentration of sodium chloride decreased more rapidly in number than ones frozen in distilled water and various concentrations of sugars (Tanner and Wallace, 1931).

When phosphate buffer at pH 7.0 (0.0003M KH_2PO_4) was used as the freeze suspending agent for Salmonella typhimurium, Staphylococcus aureus, and Streptococcus faecalis, a 90 % reduction in viable count was obtained in twenty-four hours at -21 C (Woodburn and Strong, 1960). Bretz and Hartsell (1959) obtained similar results using E. coli frozen in phosphate buffer (M/15 pH 7.0 KH_2PO_4). After freezing E. coli for seven weeks at -9 C, they obtained a 99 % reduction in viable count. On the other hand, Mazur (1960 a) found that 0.1 molar solutions of KH_2PO_4, NaCl, and $CaCl_2$, when used as suspending mediums, gave almost identical results as those obtained with distilled water.

Effect of other factors. Most investigators have not concerned themselves with studying to any extent, the more

subtle manipulations of the experiments which can effect the freezing and thawing process on microorganisms. These include such factors as : cell concentration, age of the culture, cultural conditions, pH of the suspending fluid, and aeration of the cells before freezing. It has, however, been shown that factors such as these can significantly change the results obtained after freezing and thawing.

Major et al. (1955) demonstrated with E. coli and other bacteria, that initial cell number influenced the survival after freezing at -22 C. Eretz (1961) substantiated this work using E. coli. He further stated that the greater the initial cell number, the greater the recovery.

Age of cultures used for freezing can influence the per cent recovery but this is controversial. Cultures of E. coli in the log phase of growth, were found to be more susceptible to freeze damage than those twenty-four hours old (Toyokawa and Hollander, 1956). Jones and Fabian (1952) found no difference between log phase growth cultures and older cultures.

Harrison and Cerroni (1956) and Harrison (1956) aerated suspensions of E. coli before freezing at -22 C for one week and found that the aerated cells were much more resistant than those not aerated. Eretz and Hartsell (1959) also using E. coli and freezing at -9 C for three to six months, reported that aerated cells were more sensitive to freezing.

The hydrogen ion concentration of the freeze suspending menstra is of importance. Less damage occurs to cells suspended in a medium around neutrality whereas alkaline or acid conditions favor greater reduction in viability (Tanner and Wallace, 1931; McFarlane and Gorsline, 1943; Arpai, 1962).

Other investigators have stressed the importance of using diluents of high osmotic strength such as 50 % sucrose for diluting organisms after freezing (McCleskey and Christopher, 1941; Bretz and Hartsell, 1959; Hartman and Huntsberger, 1961).

II. Theories On the Mechanisms of Freeze Death

A great many theories have been proposed to explain the cause of death and injury to microorganisms subjected to sub-zero temperatures. Much difficulty has been encountered, due to the fact that each individual set of experimental variables results in different per cent survivals. Any one of these variables could possibly change the mode of action.

Luyet and Gehenio (1940) have discussed fully in their monograph on low temperature, the theories of death. They presented a list of theories which a number of workers have used to account for the mechanism of freezing as follows: (a) A withdrawal of energy,(b) the attainment of minimal temperature, (c) mechanical injury

(d) too rapid thawing, (e) dehydration, and (f) various physiological, physical, and chemical changes. While most of these theories have been postulated for biological systems other than microorganisms, they still could apply.

Belehradek (1935) has also postulated mechanisms of freeze death and injury. They are listed as follows: (a) intracellular ice formation with resulting cell wall rupture by dilation of the freezing water, (b) cell destroyed on thawing, (c) mechanical crushing of cells by extracellular ice, (d) forming ice withdraws water from the cell causing dehydration, (e) ice forming outside the cell destroys the protoplasmic surface-layer, (f) ice is produced in the interior of the cell with a simultaneous ice formation around the cell, (g) death occurs without ice formation by direct action of cold. Again these theories pertain more to systems other than microorganisms, but they have at one time or another found wide acceptance to explain the freezing phenomenon in microorganisms.

Extracellular ice theory. Keith (1913) postulated that the mechanism of freeze death was mechanical destruction by ice crystal formation. His evidence came from the fact that milk and glycerin (5 to 42 %) when used as

suspending agents, afforded good protection to bacteria upon freezing at -20 C. Tap water did not favor survival. He believe that as the solution froze, the bacteria and non-aqueous matter were extruded from the forming ice crystals. The cells were then protected by either laying in or among these materials, thus protecting them from being crushed or otherwise injured. Tap water, on the other,hand, would have just the opposite effect. The bacteria would be crushed by the ice crystals. Conclusions drawn by Hilliard and Davis (1918) support those of Keith's, that extracellular ice formation was the mechanism.

Further evidence to support the extracellular ice theory comes from Goetz and Goetz (1938). They used Saccharomyces cerevisiae suspended in Ringer's solution and frozen at different temperatures. Freeze death in this instance, was termed physical death. Physical death and ice crystallization were found to be definite functions of each other--by suppressing crystallization, the death rate could be controlled.

Weiser and Osterud (1945) suggest strongly that the "immediate death" occurring when microorganisms are frozen is due to extracellular ice formation. By using freezing times of only eighty seconds with E. coli sus-

pended in 1.0 % peptone, pH 7.0, and visually observing ice formation, they found the greatest decrease in viable cell counts to occur during formation of ice.

Proom and Hemmons (1949) stated that extracellular ice crystals punctured the cell wall and membrane. Their results revealed that Staphylococcus aureus, E. coli, and Shigella dysenteriae survived much better when in saline and broth at -78 C, than Vibrio comma, Neisseria intracellularis, and Neisseria gonorrhoeae. Differences in survival between these two groups of organisms, they postulated, was due to the strength of cell wall. In fact, they did find many degenerative forms on microscopic examination.

The evidence against extracellular ice as the mechanism of freeze death and injury is just as impressive. But it must be kept in mind that experimental conditions are not the same, therefore possibly resulting in different mechanisms. The most striking evidence that extracellular ice does not mediate death and injury to microorganisms, was recently presented by Bretz (1961). By freezing E. coli at -9 C on cellophane or membrane filter disks and comparing survivals after freezing to cells suspended in M/15 phosphate buffer and treated in the same manner, he found that the results were the same.

Since extracellular ice would not be present on the filters, it can be ruled out as the cause of death.

Harrison and Cerroni (1956) presented data correlating physical strength and susceptibility to freezing to disprove extracellular ice and its crushing effect. Two test organisms, E. coli and Microbacterium flavum were employed. They made two suspensions of each organism; one was frozen and thawed a number of times, the other being blended in a tissue disintegrater. E. coli proved to be more susceptible to freezing and thawing than M. flavium, though E. coli was physically stronger, as shown by the disintegrater experiments. From these data, they drew the conclusion that there was no correlation between the physical strength of the cells and their susceptibility to freezing. On the basis of this evidence, they further concluded that the lethal factor of freezing and thawing was not mechanical.

Several investigators, Haines (1938) and Jones and Fabian (1952), have shown that different species of bacteria did not change in size after freezing nor could they find any broken or distorted cells. If ice crystals did crush cells, these changes should have been evident.

Weiser and Hargiss (1946) designed an experiment to prove or disprove the mechanical ice crushing theory.

The test organism, E. coli, was suspended in a 10 % sucrose solution. Only 0.02 ml. of the suspension was placed between two cover slips and frozen in liquid nitrogen. This rapid freezing causes the formation of a vitreous state. Thawing was then carried out in one of two ways in order to bring about either devitrification or vitromelting. The control was a sample which was allowed to crystallize. It was expected that the vitrifying and vitromelting would cause the least damage, and crystallization and devitrification, the most damage. However, it was found that crystallization caused the least damage, indicating that extracellular ice formation was not as important as previously thought.

It is well known that cold shock can injure microorganisms (Hegarty and Weeks, 1940; Meynell, 1958). This implies that death may be due to the effect of cold alone, and mediates to an extent, against the mechanical death theory. However, Wieser and Osterud (1945) found that upon supercooling suspensions of E. coli, reductions of only about 3 % , as opposed to reduction of about 50 % for freezing occurred. Mazur (1960 a, 1960 b,) also found this to be true. Harrison (1956), using several different organisms suspended in 4.6 M sodium chloride, got good reductions upon supercooling.

The difference probably lies in the fact that Weiser and Osterud used a different suspending agent than Harrison.

Intracellular ice theory. Intracellular ice formation has often been postulated as being the major factor in freeze damage to microorganisms. Due to the small size of the organisms, all evidence to date has been by necessity, circumstantial.

Mazur (1960 a, 1960 b) has been the main proponent of intracellular ice formation in microorganisms as the chief cause of death and injury. Evidence for this, he believes, comes from the fact that the organisms he used have a definite temperature range, where injury is the greatest. According to his theory, extracellular ice is a prerequisite for intracellular ice formation. He suggests that water within the cell has a tendency to supercool, possibly due to the fact that there is little likelihood for nucleation centers to form in very small volumes of water found in the cell. But when extracellular ice appears in the external medium, it enters the cell, acts as a nucleation center for the supercooled water, and effectively seeds it, causing freezing. This explanation requires further hypotheses.

First it must be assumed that ice crystals can enter into the cell. This, he feels, is accomplished by the fact that the cell membrane and wall have small water-filled channels only angstroms in diameter. Ice crystals must have a sufficiently small radius of curvature in order to pass through these channels and seed the intracellular water.

Since it is known that the equilibrium freezing or melting point of a crystal of such a critical radius would no longer be 0 C, but at a much lower temperature, possibly even -30 C (Chambers, 1959), it fits the data which Mazur has collected. This would explain why rapid cooling is so lethal as opposed to slow cooling. Small ice crystals form when rapid cooling takes place, and these are small enough to pass through the pores. Slow cooling would favor much larger crystals and hinder passage. Nei (1960) has reported photographing intracellular freezing in rapidly cooled yeast cells but not in slowly cooled ones.

Calorimetric measurements on populations of yeast cells that allowed determinations to be made on the fraction of cellular water frozen as a function of temperature, have been made (Wood and Rosenburg, 1957). Determinations showed that normal yeast cells contain

69 % total water. As the temperature was lowered below 0 C, a progressive amount of this was frozen until at -22 C, about 87% of the total water was frozen.

Weiser and Osterud (1945) presented evidence against the theory that ice forms within bacterial cells. Their first finding in support of this, was that the intensity of the freezing temperature has no influence on "immediate death" due to freezing of E. coli in 1.0 % peptone. If intracellular ice were to contribute a lethal effect, there should be some temperature where the lethality was more pronounced. Repeated flucuations of the suspensions between the temperature ranges of -1.5 C to -195 C, did not cause any more damage than that in stored controls. Finally it was observed that marked "immediate death" occurred at temperatures just below 0 C, where intracellular ice would not be expected to occur.

More direct evidence against intracellular ice comes from Haines (1938) and Jones and Fabian (1952). By staining cells after freezing, neither could find any distorted cells which would be expected to occur in a population, if extensive damage due to the intracellular or extracellular mechanical action of ice had occurred.

The withdrawal of water from cells, due to the formation of the extracellular ice and the bound water present in cells (Luyet and Gehenio, 1940), mediates against intracellular ice formation. The various eutectic points of the protoplasm of microbial cells would also offer protection against intracellular freezing at higher temperatures.

Luyet (1951) could find no evidence of intracellular ice formation in frozen cells of Streptococcus lactis examined with the electron microscope. His criterion for intracellular freezing was comparison of cell size and cytoplasmic granule disturbance to unfrozen cells.

Concentration of solutes theory. As previously stated, most investigators who have used distilled water to suspend microorganisms for freezing, have found it not too detrimental. Recoveries have usually been between 90 and 99 %, based on viable cell counts. However, if certain solutes or electrolytes are added to distilled water, the per cent survivals are in many cases, decreased. This phenomenon has been attributed to the concentration of these solutes or electrolytes around the cells during freezing. As water freezes it extrudes this foreign matter, leaving it un-

frozen until the various eutectic points are reached.

Haines (1938), in his classical work on the effect of freezing on bacteria, found that -2 C was the most injurious storage temperature. Working with Pseudomonas aeruginosa, he isolated the native cellular protein by low temperature extraction methods. Upon freezing the protein at -2 C, he found that one fraction rapidly coagulated. Such flocculation was negligible at -20 C. This lead him to postulate two factors being responsible for bacterial freeze death: one unknown but apparently not mechanical, and the other causing the flocculation of cellular protein. He thought it was possible that the flocculation was due to concentration of solutes or change in pH.

More recent work with E. coli and Lactobacillus fermenti suspended in broth, broth diluted ten-fold, one-hundred-fold, and distilled water with freezing at -22 C, has shown that per cent recoveries are higher, the more dilute the broth (Harrison, 1956). Undiluted broth gave the smallest per cent survival, whereas distilled water gave the highest. By using 4.6 M sodium chloride which remained unsolidified at -22 C, the survival curves had a continuously negative slope, indicating that solute concentration was probably respon-

sible. Taking advantage of the fact that glycerol protects E. coli when frozen, he designed an experiment using two different suspending agents: 4.1 M glycerol, and 1.4 M sodium chloride. Cooling was carried out at -22 C for one-and-a-half hours without ice crystal formation. Glycerol alone, caused no reduction. Sodium chloride (1.4 M) produced 60 % reduction but the glycerol-sodium chloride mixture caused only 26 % reduction. It therefore seems that glycerol acts as a solute buffer, protecting cells from high concentrations of solutes since it can counteract much of the damage induced by the sodium chloride.

Further work along this approach by Reeves and Harrison (1957) supported their previous work. They found that cells which are able to survive the rapid decline in viability at -22 C in 4.6 M NaCl, are able to maintain themselves in it for long periods, but this resistance is not heritable.

Both Lovelock (1957) and Meryman (1956), who have worked extensively with the effect of freezing on red blood cells and mammalian cells, believe the damage is due to concentration of electrolytes.

There are reports in the literature which provide evidence that high concentration of solutes and their

other effects are not operative under certain circumstances.

As was stated before, Pretz (1961) froze bacteria on cellophane and membrane filters. Results indicated that reductions were as low as those for organisms suspended in 0.067 M phosphate buffer. Since organisms impinged on cellophane and membrane filters are not in a liquid medium, this eliminates the possibility of concentration of solutes.

Mazur (1960 a, 1960 b) has spoken out against high concentration of solutes as the major cause of damage in freezing. With Saccharomyces cerevisiae suspended in 0.1 M solutions of KH_2PO_4, NaCl, and $CaCl_2$, he found the per cent survivals compared favorably with those of distilled water suspended organism after freezing. He also stated that if death were the result of high concentration of solute, one would expect longer exposures to produce greater damage. He found this to be just the opposite.

In an experiment similar to Harrison's (1956), Mazur (1960 b) used Asperigillus flavus spores suspended in unfrozen and frozen solutions of 4.0 M calcium chloride and 3.3 M $MgCl_2$. If the media were to be kept in an unfrozen state, the recoveries would be high. However, if

they were frozen, recoveries were very low. This seems to mediate against high concentration of solutes in this particular system.

Metabolic damage theory. It is possible that the freezing process severely stresses microorganisms, causing injury which is manifested by metabolic damage. This damage could appear in many forms which would progressively degrade the cell until death resulted.

Currans and Evans (1937) and Nelson (1943) drew attention to the fact that bacteria and their spores are more demanding in their nutritional requirements after being subjected to extremes of physical or chemical environment. Similar reports have shown that the same is true for freezing. Gunderson and Rose (1948) found that violet-red bile agar is very inhibitory to coliform organisms found in foods. Goresline (1946) reported the glucose tryptone agar was superior to other plating media in the recovery of organisms from frozen vegetables. Hartsell (1951 a) did further work on this with E. coli, Shigella dysenteriae, and Micrococcus aureus suspended in a preparation of egg-culture mixture and freezing at -9 C and -18 C for up to a year. He found that E. coli and S. dysenteriae grew better on yeast extract-veal infusion agar, than on MacConkey agar

after freezing. Higher recoveries were also found on YE-VI agar, than on Staphylococcus 110 agar when M. aureus was frozen in the same manner. However, if the defrosted organisms were allowed to stand for one to six hours, no inhibition was found upon plating them on their respective selective media. The reason for better recoveries on YE-VI agar, he felt, was due to certain vitamins and trace elements, plus the fact that it contained no selective, toxic agents.

A similar publication by Hartsell (1951 b) also showed that such selective media as desoxycholate, violet red bile, MacConky and Staphylococcus 110 agars, were inhibitory to bacteria after they have been frozen in beef and peas. YE-VI agar was again used as a standard reference medium. He is of the opinion that the freezing process like the heating process in foods, alters the nutritional requirements of bacteria. If then, these cells are given a booster dose of metabolites, they can be recovered.

Evidence of this nature strongly suggests that the cells are in some way injured or damaged. Dubos (1937) showed that pneumococci, living or dead, to be liable to lysis, must have their autolytic enzymes activated. Freezing accomplished this activation. Haines (1938)

found that the protein of Ps. aeruginosa could be denatured by freezing, indicating possible injury, depending upon the extent of damage. Turner and Frayton (1939) have suggested that freezing of microorganisms at temperatures in the vicinity of -10 to -20 C, could bring about injury mainly be changes incident to cell metabolism or to proteolytic or other enzymes.

The injury to microorganisms can be progressive, changing unharmed cells to injured, with varying degrees (Straka and Stokes, 1959). Using this criterion, Straka and Stokes found that this was true. By using a synthetic medium and a complete medium (trypticase soy agar), they were able to show that E. coli and several species of Pseudomonas were metabolically damaged after freezing at different temperatures and times. This metabolic damage was manifested by the fact that the complete medium could recover more organisms than the synthetic. The per cent of injured cells in a population after freezing, was determined quantitatively by the difference in plate counts on the two media. Injury varied with the time and temperature of storage, and with the nature and pH of the suspending fluid. The addition of 2 % trypticase to the synthetic agar increased recoveries after freezing, to those comparable with

trypticase soy agar. Other substances which did not prove active included acid hydrolyzed casein enriched with cystine and tryptophan, mixtures of B-vitamins, purines, and pyrimidines. They suggest that the activity was due to the presence of peptides in the trypticane.

Work with Shigella sonnei by Nakamura and Dawson (1962) has shown the same type of freeze injury. Cells frozen in saline exhibited greater injury than those frozen in nutrient broth or milk. By addition of meat extract, peptone, or casamino acids to their synthetic medium, recoveries were again brought to the level of those on nutrient agar and brain-heart infusion agar containing whole human or rabbit blood.

Arpai (1962,1963) also using a comparison between a complete medium and synthetic medium to determine freeze damage, found that as the temperature was lowered from -7 C to -18 C or -30 C, the decrease in the number of unharmed cells was much less rapid. He also found that the number of injured cells increased as the killing rate decreased. Using motility as a criterion of freeze damage, he found that it could be correlated to non-lethal metabolic freeze injury and further, injury was effected by the time and temperature of storage as well

as the nature of the suspending fluid.

Moss and Speck (1963), using Streptococcus lactis, have reported metabolic damage due to freezing, using techniques similar to those just discussed.

Cellular leakage and its consequences. At the present time, it is currently acceptable to speak of "leaky bacteria" (Hartsell, 1959) and cellular effluent (Harrison, 1961). Leakage has been found to occur not only in frozen cells but in cells which have been stressed in other ways. There also seems to be an interrelationship between cellular leakage and cell metabolism. It has been further reported that the leakage material and other substances can stimulate or restore cellular activity, depending on the criterion used. At the present time, this concept offers a plausible explanation for freeze injury and death and perhaps other types of cellular injury and death.

Hartsell (1951 a, 1951 b) and Squires and Hartsell (1955) reported that organisms frozen and thawed, initiated more rapid growth than those which were not frozen. Tanguay (1959) also found this greater growth response with several of his test organisms. He indicated that it was possible that this could be due to accumulation of stimulating materials within the cell

during frozen storage.

Further elaboration on this theory came from Hartsell (1959) as follows: "Storage at subfreezing temperatures creates a special type of dormant state in nonsporulating species. A synthesis, even at subfreezing temperatures, might allow substance A to be utilized in product C, but the use of product C to form D, which latter product is essential to cellular division, could not occur until the cells were defrosted. If this conception of events is true, product C should accumulate as the cells are held in storage and could be isolated if appropriate techniques were available."

The isolation of product C was reported by Hartsell (1961). It is called "Factor S" - - for stimulating. It can be isolated from E. coli suspended in Sorenson's buffer for fifty-five days at -9 C; as yet it has not been identified. The yields of the factor are very low. It can stimulate other bacteria, and it appears not to be a vitamin, amino acid, or a carbohydrate. Mehler and Hartsell (1963) developed an assay procedure for "Factor S" activity by observing spectrophotometrically, its stimulating influence on the growth rate of homologous and/or heterologous broth cultures. Other organisms which were found to produce "Factor S" were

Pseudomonas fragi, Sarcina lutea, Staphylococcus aureus, Bacillus coagulans, Bacillus stearothermophilus.

Deotto (1944) also observed a stimulation in cold shocked E. coli. Using warburg techniques, he found that if the cells were placed at 0 C for 20 to 30 minutes, they showed an increase in oxygen uptake after being placed back in the water bath at 38 C. It was thought that stimulation was independent of multiplication of the cells and dependent upon the liberation of a respiratory stimulant from the cold-shocked cells.

Bretz (1958), attempting to characterize the properties of the leakage material from E. coli frozen at -9 C in phosphate buffer for various periods, found no release of ultraviolet-adsorbing substances, but ninhydrin-positive materials were detected. Later, Bretz and Basa (1960) demonstrated that the loss of this material from E. coli in M/15 phosphate buffer stored at -9 C, protected the survivors. They also showed that this material adsorbed to the cells. Characterization of this material was carried out (Ambrosini and Bretz, 1963) by dialysis, ashing, and chelation experiments. It was shown that the protective factor was not a metal, or small molecules. Norite treatment and dialysis demonstrated that large

and small molecular weight compounds were present. The large molecular weight compounds gave protection, while the smaller molecules were actually inhibitory.

Other examples of leakage material from microorganisms have been reported in the literature. Holden (1958) found that nucleotides leached out of Lactobacillus arabinosus during incubation in phosphate buffer at 37 C. These nucleotides came from the degradation of intracellular nucleic acids. Ultra-violet absorbing substances were detected in cell leakage material from Bacillus megatherum suspended in phosphate buffer for two hours at 37 C (DeLamater et al., 1959). Two fractions were detected, one dialyzable and the other not dialyzable. Washed cells of Saccharomyces cerevisiae, incubated in .08 M sodium citrate for two hours with 2 % glucose added, leached material (Higuchi and Uemura, 1959). The supernatant contained ultraviolet absorbing substances with a maximum at 258 mu and a minimum at 235 to 240 mu. The nucleotides were probably fragments of ribonucleic acid. By freezing and thawing the cells, the supernatant was found to contain a greater quantity of material. However, they felt that not all of this material might be the same since an increased absorption at 230 to 250 mu was noted.

Little work has been done with the leakage material from frozen cells as far as their effect upon cellular metabolism and restoration of activity. Work has been done with restoring the viability of cells exposed to ultra-violet light, heat, and chlorine (Hemmets et al., 1954 a, and 1954 b), using various metabolites.

Lund (1961) incubated frozen washed yeast cells in supernatant collected after freezing and in distilled water in Warburg vessels. With the frozen cell supernatant, an 86 % increase in cellular nitrogen was found as opposed to only a 5 % increase in distilled water. Leakage of phosphorus into the suspending medium was detected also.

Povernick (1957) and Povarnick and Allen (1954) found that typhus rickettsiae frozen in isotonic salt solutions, showed a greatly decreased toxicity for mice, hemolytic activity, respiration, and infectivity for eggs. Nicotonamide adenine denucleotide could restore most of this activity and coenzyme A, part of the activity. NAD was detected in the suspending medium after freezing.

It has been postulated that freezing causes an altered permeability of the cell membrane or wall. Changes such as these would permit the loss of cellular

constituents and perhaps the entry of some previously impermeable substance.

The term, osmosensitiveness, has been applied to changes in cell wall and membrane of frozen organisms upon freezing (Dretz and Hartsell, 1959). They found That 10 % sucrose was a better diluent for frozen organisms than distilled water or various phosphate buffers. The high osmotic strength of the sucrose diluent protected the frozen organisms by creating a more favorable environment and perhaps stopped leakage from these cells even after freezing.

Lipid-protein complexes of cell membranes could possibly be ruptured upon freezing since they are not held together by strong covalent bonds, but by weak association forces (Lovelock, 1957). Causes of this could be increase in electrolyte concentration changes in pH, and removal of water. His work with red blood cell membranes, proved that the cells lose phospholipids when suspended in various molar solutions of NaCl.

Lysozyme is known to attack cells which in some way have been stressed by heat, freezing, etc. Kohn and Szybalski (1959) and Kohn (1960), working with E. coli, found that the cells were insensitive to lysozyme in the growing or resting cell states. However, frozen and

thawed organisms were sensitive to lysozyme. They postulated that freezing and thawing tore open the outer layer of the cell wall, which is believed to be a plastic lipoprotein layer and resistant to lysozyme. Restoration of lysozyme insensitivity occurred in about 30 minutes after freezing and could not be stopped by such inhibitors as chloraphenicol. This implies that some type of repair process is going on in these cells due to the damage caused by freezing.

Other permeability alterations have also been noted. Baker's yeast, when unfrozen, does not adsorb or metabolize citrate but once it has been frozen, citrate becomes freely permeable but still is not metabolized (Foulkes, 1954).

MATERIALS AND METHODS

Cultivation of organism: Escherichia coli (ATCC # 11303) obtained from the Division of Laboratories, Michigan Department of Health, was used throughout the work. Cells were grown on tryptone glucose extract agar slants for 18 hours at 37 C and harvested with the proper diluent. The pooled cells were placed in sterile centrifuge cups, centrifuged at 12,100 x g for 20 minutes, and washed three times with the proper diluents. Sufficient diluent was then added to give a final concentration of about 2.0×10^{10} organism per ml, with a dry weight of about 6.0 mgm per ml. After the cells were thoroughly mixed, serial dilutions were made using Butterfield's Buffer (Butterfield, 1933). Duplicate TGE agar pour plates were made and incubated at 37 C for 24 hours before counting.

Suspending fluids. Four different suspending fluids were used to suspend the organisms for freezing: (1) distilled water, (2) 0.85 % NaCl, (3) 0.15 M phosphate buffer (10.65 g. Na_2HPO_4, 10.25 g. $NaH_2PO_4 . H_2O$, 1000 ml distilled water) pH 6.8 ionic strength 0.12, and (4) 0.06 M phosphate buffer (4.26 g. Na_2HPO_4, 4.14 g. $NaH_2PO_4 . H_2$), 1000 ml distilled water) pH 6.8 and ionic strength 0.30. Hydrogen ion concentration determina-

tions were made on the distilled water and 0.85 % NaCl. In most instances, the pH was about 6.8. For this reason, the phosphate buffers were made to pH 6.8.

Freezing and Storage. Five ml portions of the cell suspensions were pipetted into sterile 20x150 mm glass test tubes for freezing. Freezing and storage of the cell suspension were carried out in a cooling bath containing a mixture of 95 % ethanol and water placed in the freezing compartment of an ordinary refrigerator. The temperature of the bath was -16 C ± 1. A frequent check was made on the temperature of the bath with a thermometer, and a Micromax Automatic temperature recorder (Leeds and Northrup Co., Philadelphia, Pa.). The glass test tubes were placed in a slanted position to avoid breakage. From 5 to 7 minutes was required for freezing of the 5 ml portions of the cell suspensions. Storage time was 22 hours.

At the end of a storage period, the tubes were removed from the freeze bath and thawed by immersion with gentle agitation, in a 50 C water bath. About 90 seconds were required to thaw the suspension and reach room temperature. The cells were then pooled in sterile centrifuge cups and mixed. Determination of viable cells was made as previously described.

Preparation of cells for oxygen uptake experiments.

In all experiments, 0.5 ml of the resting cell suspensions, was added to the Warburg vessels. Three different preparations of the cells were used in the Warburg apparatus, depending upon the experiment:

(1) unfrozen cells prepared as previously described

(2) cells frozen and thawed just prior to use (These will be termed "frozen cells")

(3) cells frozen and thawed, separated from their supernatant by centrifugation, and resuspended in the same type of fresh diluent (These will be termed "centrifuged cells")

The procedure for the preparation of (2) and (3) was as follows: From 3 to 5 ml of the pooled frozen cells in the centrifuge cups were removed. The remaining cells were centrifuged in the cold for 30 minutes at 22,000 x g. The resulting supernatant was decanted and the volume noted. This represents the frozen cell supernatant used in the oxygen uptake stimulation experiments. An equal amount of the proper diluent was added to the cells in the centrifuge cups, followed by thorough mixing. Viable cell determinations were carried out on these cells to ascertain if the proper cell concentration had been maintained.

Manometric techniques. The usual Warburg techniques were employed to determine the oxygen uptake of the cell

suspensions (Umbreit, et al., 1957). The contents of the flasks were: 0.5 ml of the resting cell suspensions, 0.5 ml of 0.067 M phosphate buffer pH 7.0, with 0.2 ml of 20 % KOH in the center well. In all experiments except one, 0.5 ml of 0.05 M glucose ("Difco" Bacto-dextrose) was placed in the side arm as substrate. To determine whether or not the cells had undergone a permeability change, 0.5 ml of 0.05 M sodium citrate was used as substrate, either alone, or with 0.1 ml of 0.01 M sodium acetate. The volume of the vessels was brought up to 3.0 ml by the addition of distilled water.

The frozen cell supernatants from each of the four freeze suspending agents were tested against their respective centrifuged cells by adding 1.3 ml of the supernatant to the vessels in place of distilled water, 0.85% saline, and 0.15 M phosphate buffer were tested by addition of 1.3 ml to the vessels as follows:

(1) frozen cell supernatant from distilled water suspended cells, tested with centrifuged cells which had been frozen in 0.85% saline and with centrifuged cells which had been frozen in 0.15 M phosphate buffer.

(2) frozen cell supernatant from 0.85% saline suspended cells, tested with centrifuged cells which had been frozen in distilled water and with centrifuged cells which had been frozen in 0.15 M phosphate buffer.

(3) frozen cell supernatant from 0.15 M phosphate buffer suspended cells tested with centrifuged cells which had been frozen in 0.85 % saline

and with centrifuged cells which had been frozen in distilled water.

Various cofactors were added to the reaction mixture in the vessels using centrifuged cells with no frozen cell supernatant added, to determine whether or not, they could simulate the stimulating effect. The cofactors were added to the vessels in 0.1 ml portions, giving a final concentration of each as follows: 2 um nicotinamide adenine dinucleotide, 2 um adenosine triphosphate, 2 um adenosine diphosphate, and 10 um $MgCl_2$. These were either freshly made for each experiment or maintained in the frozen state.

The flasks were equilibrated for 15 minutes, and the oxygen uptake was followed for a period of 50 minutes at 37 C. All suspensions were tested in duplicate. The results reported, represent values after subtracting the endogenous rates.

Characterization of frozen cell supernatant. In order to determine some of the properties of the frozen cell supernatant from cells frozen in saline, various procedures of characterization were employed. In all instances the activity of the treated supernatant was compared against untreated supernatant, using centrifuged cells for each.

Dialysis was used to determine the diffusibility of the active material. Ten ml of the frozen cell supernatant was placed in cellulose tubing and dialyzed against 0.005 M phosphate buffer in the cold for 24 hours.

Heat stability was determined by heating 10 ml of the supernatant at 100 C for 30 minutes. The heated supernatant was then rapidly cooled under cold tap water.

An adsorption experiment was performed using an activated charcoal, Norit A (Pfanstiehl Chemical Co., Waukegan, Ill.). One-tenth gm of Norit A was added to 10 ml of supernatant. After mixing, the supernatant was incubated at 37 C to increase the adsorption. The charcoal was removed from the supernatant by gravity flow filtration.

Spectrophotometric techniques. A Beckman Model D U spectrophotometer was used for all spectrophotometric work. Three ml of saline frozen cell supernatant was added to quartz cuvettes and the absorption spectrum noted.

Glucose dehydrogenase isolated from Bacillus cereus (courtesy of Mr. John Kools), was used to assay for nicotinamide adenine dinucleotide (NAD) in the saline frozen

cell supernatant. The reduction of nicotinamide adenine dinucleotide was followed spectrophotometrically at 340 mu. One cuvette contained 0.3 ml of glucose (300 um), 0.3 ml of NAD (6um), 0.3 ml of glucose dehydrogenase, 1.0 ml of 0.15 M tris (hydroxymethyl) aminomethane (tris) buffer pH 8.0, and 1.1 ml of 0.85 % saline to give a final volume of 3.0 ml. The other cuvette contained the same materials except that NAD and 0.85 % saline were excluded and 1.4 ml of saline frozen cells supernatant added in place of them, giving a final volume of 3.0 ml. A control was also run to determine whether or not the saline frozen cell supernatant might inhibit the enzyme. This was done by replacing the 1.1 ml of 0.85 % saline with an equal amount of saline frozen cell supernatant. The change in optical density at 340 mu was followed for 180 seconds.

RESULTS

Cells frozen in saline. Freezing cells in 0.85 % saline always resulted in a large reduction in viable cell counts and a large decrease in oxygen uptake. Figure 1 shows the decrease in oxygen uptake after freezing. An oxygen uptake of 298 ul was observed before freezing, as opposed to only 132 ul taken up after freezing. This represents about a 55 % decrease in uptake. Three other trials gave similar results.

The per cent recovery of cells for this experiment was 20. Variation in per cent recoveries was found for three other trials. They ranged from 11.0 % to 20.0 %.

The removal of the frozen cell supernatant from the cells caused a further decrease in oxygen uptake, from 132 ul to 110 ul (Fig. 1). By addition of 1.3 ml of frozen cell supernatant to the Warburg vessels containing centrifuged cells, the oxygen uptake could be raised to 158 ul, indicating that the supernatant possibly contained stimulating substances. The frozen cell supernatant in the presence of glucose, showed no oxygen uptake, nor was saline alone found to cause any stimulation. This was also found to be true for the other frozen cell supernatants.

The addition of 1.3 ml of supernatant to the centrifuged cells was chosen because frozen cells, which

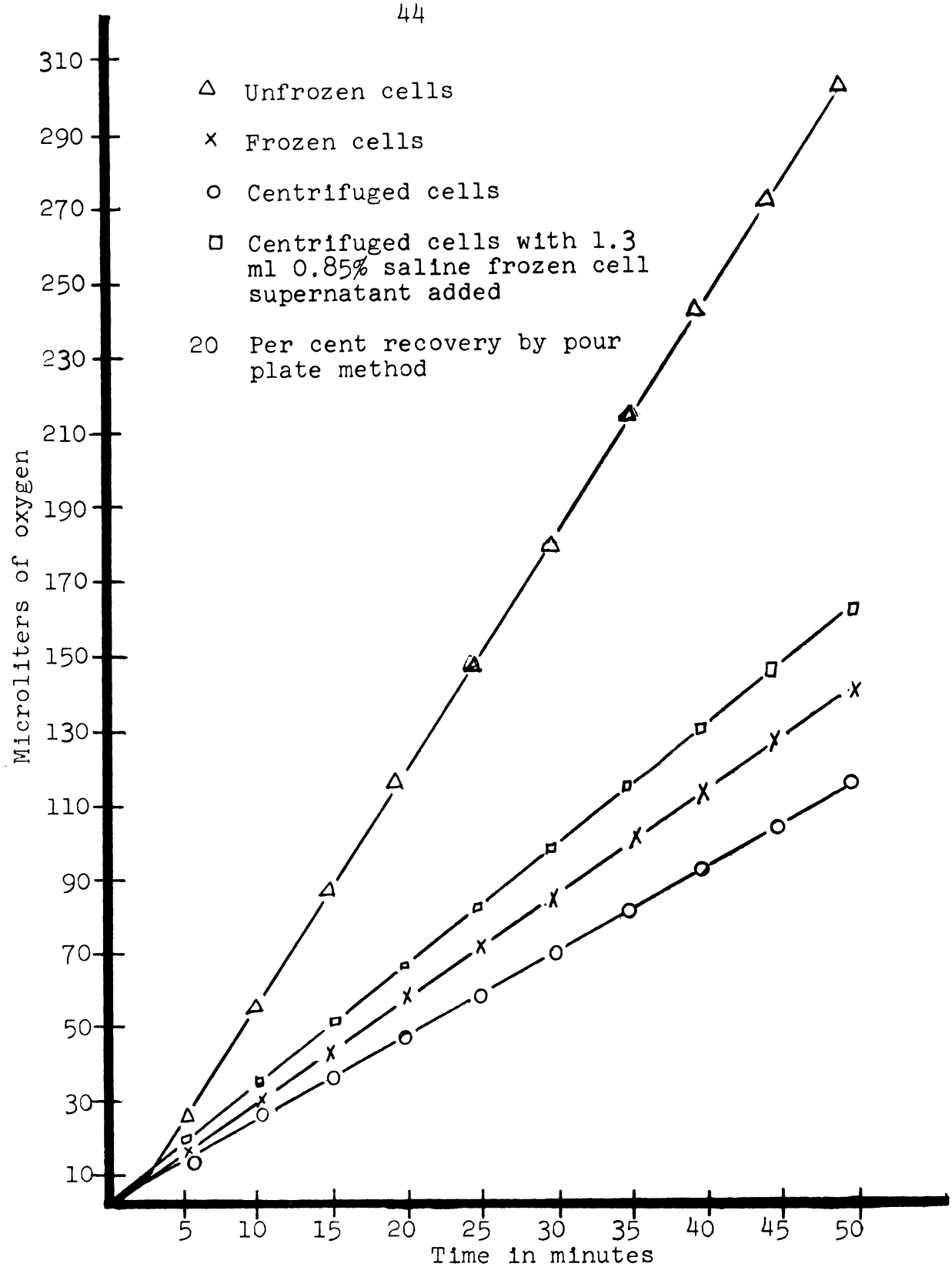


Fig. 1. Oxygen Uptake of Escherichia coli Cells Frozen in 0.85% Saline

contain about 0.5 ml of frozen cell supernatant, did not show the best stimulation, In some instances (Fig 2), the stimulation was almost the same. In no instance did the frozen cells have a greater oxygen uptake than centrifuged cells with 1.3 ml of supernatant.

Cells frozen in 0.06 M phosphate buffer. The decrease in oxygen uptake after freezing in 0.06 M phosphate buffer was 60 ul (Fig. 3). Removal of the frozen cell supernatant caused a further decrease in oxygen uptake, from 230 ul to 164 ul, a loss of 66 ul. The addition of 1.3 ml of the 0.06 M phosphate buffer frozen cell supernatant to the centrifuged cells, restored the activity to its original level of about 230 ul. However, the addition of 1.3 ml of supernatant caused no greater increase than 0.5 ml of supernatant. Two more subsequent trials give similar data to that in Figure 3.

The per cent recovery of frozen cells for this experiment was 36 %. In the other two trials, the per cent recoveries were 35 and 41.

Cells frozen in 0.15 M phosphate buffer. Freezing cells in 0.15 M phosphate buffer also caused a decrease in the respiration rate. After freezing, only 230 ul

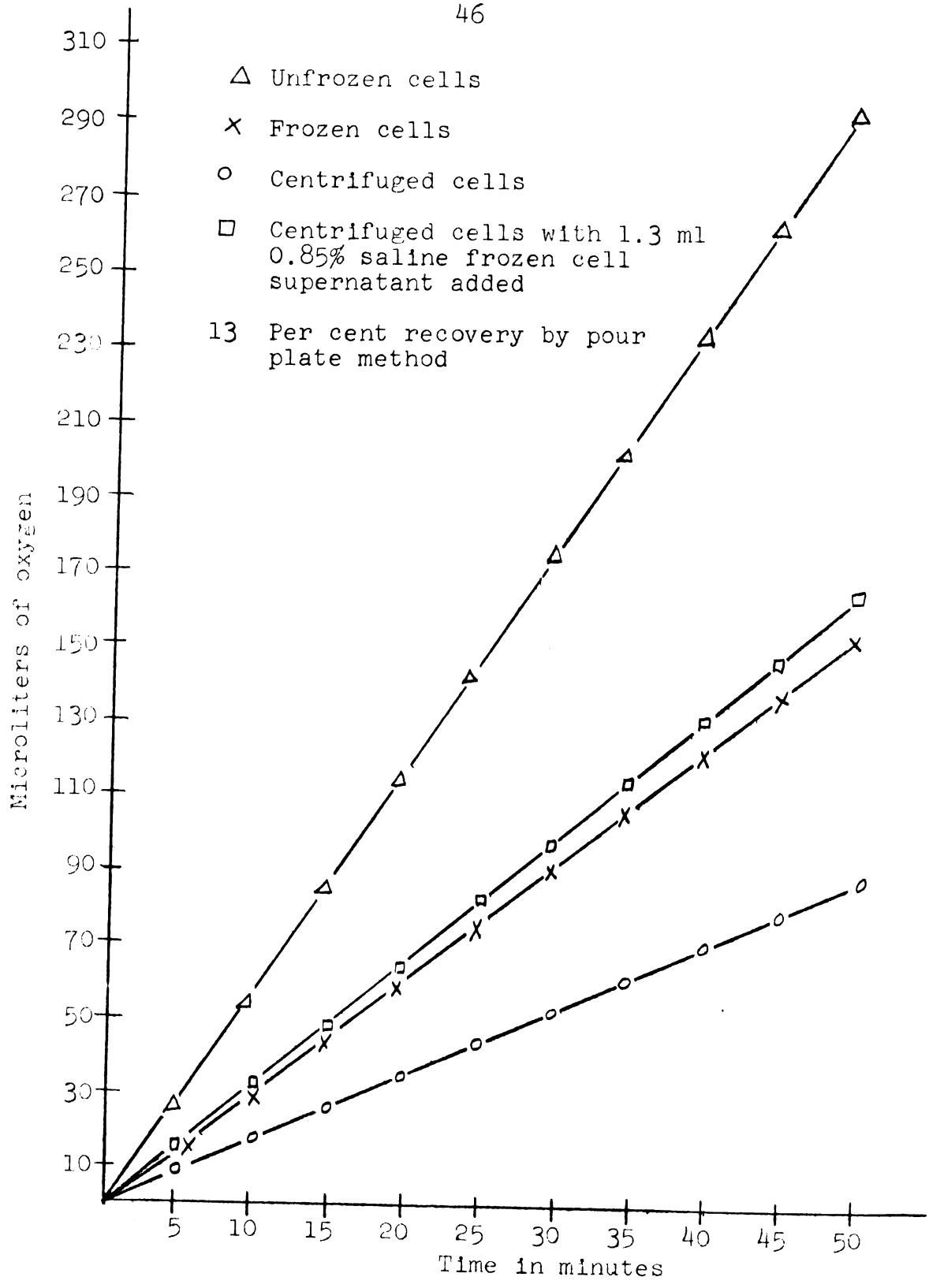


Fig. 2. Oxygen Uptake of Escherichia coli Cells Frozen in 0.85% Saline

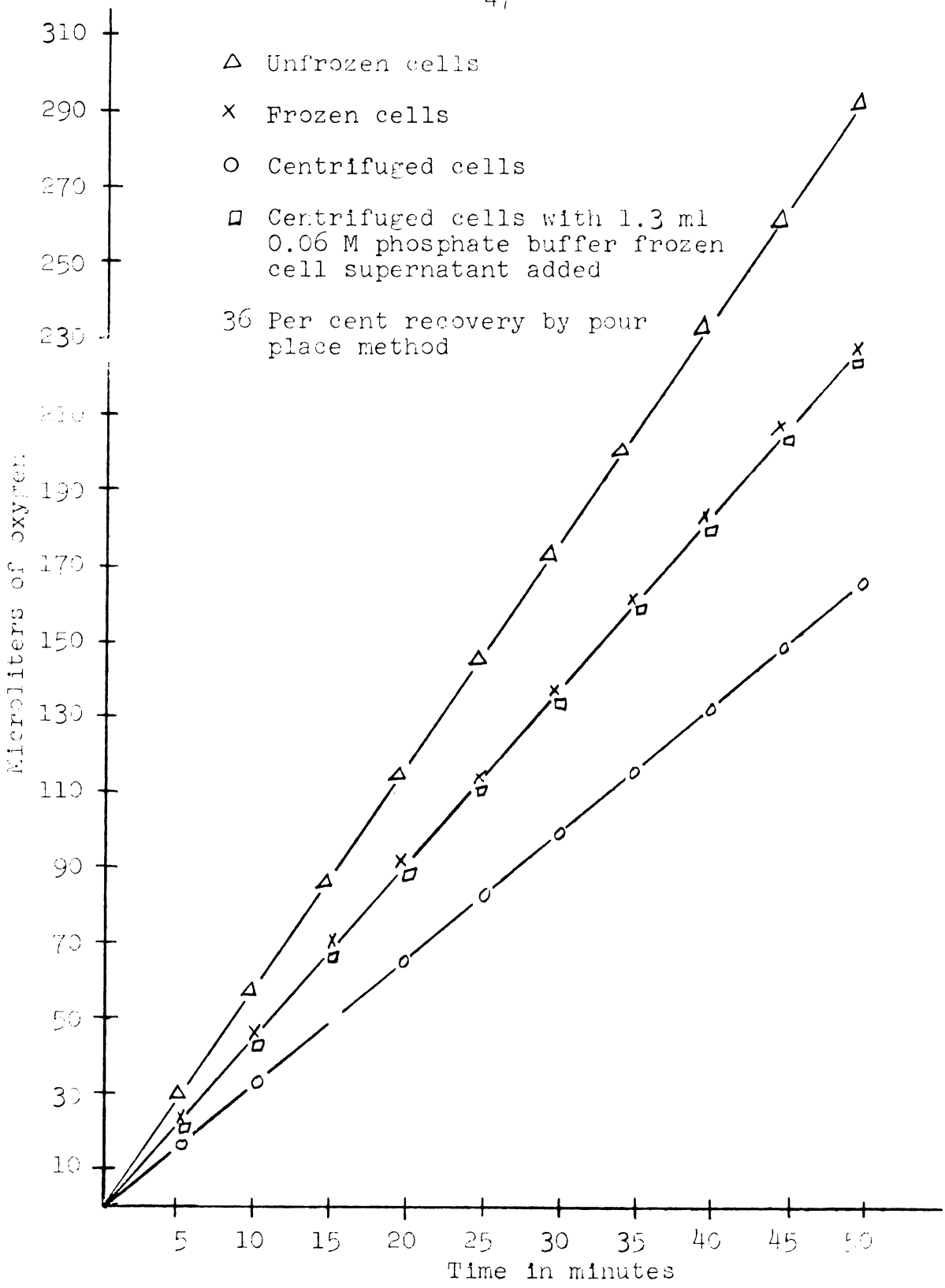


Fig. 3. Oxygen Uptake of Escherichia coli Cells Frozen in 0.06 M Phosphate Buffer

of oxygen was taken up as compared to 287 ul for the unfrozen cells (Fig. 4). This compared well with that obtained for 0.06 M phosphate buffer. When the 0.15 M phosphate buffer frozen cell supernatant was removed, a decrease of only 25 ul was noted (Fig. 4), which could be replaced to the same degree by either 0.5 ml or 1.3 ml of the 0.15 M phosphate buffer frozen cell supernatant. It therefore differed from cells frozen in 0.06 M phosphate buffer, which had a decrease in oxygen uptake of about 2.5 times this much when their frozen cell supernatant was removed.

The number of cells recovered after freezing in 0.15 M phosphate buffer for this trial, was 60 %. Two subsequent trials gave similar results. This was almost twice the number recovered from 0.06 M phosphate buffer.

Cells frozen in distilled water. Cells frozen in distilled water survived with the least damage as compared to all other suspending fluids. A decrease of only 43 ul of oxygen occurred after freezing (Fig. 5). More significant was the observation that removal of the frozen cell supernatant, or addition of 1.3 ml to centrifuged cells, caused no change in oxygen uptake. Also 86 % of the cells could be recovered after freezing.

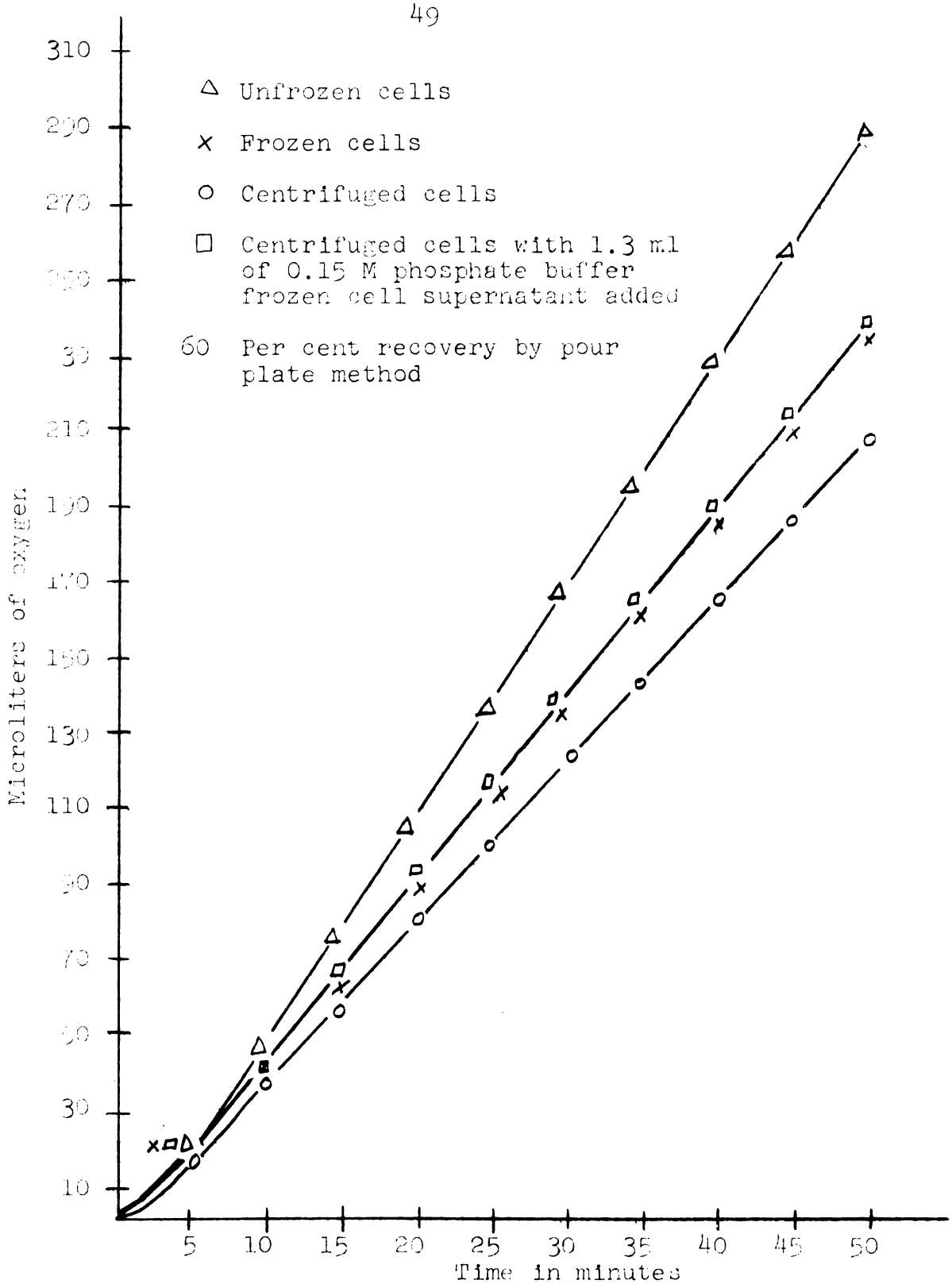


Fig. 4. Oxygen Uptake of Escherichia coli Cells Frozen in 0.15 M Phosphate Buffer

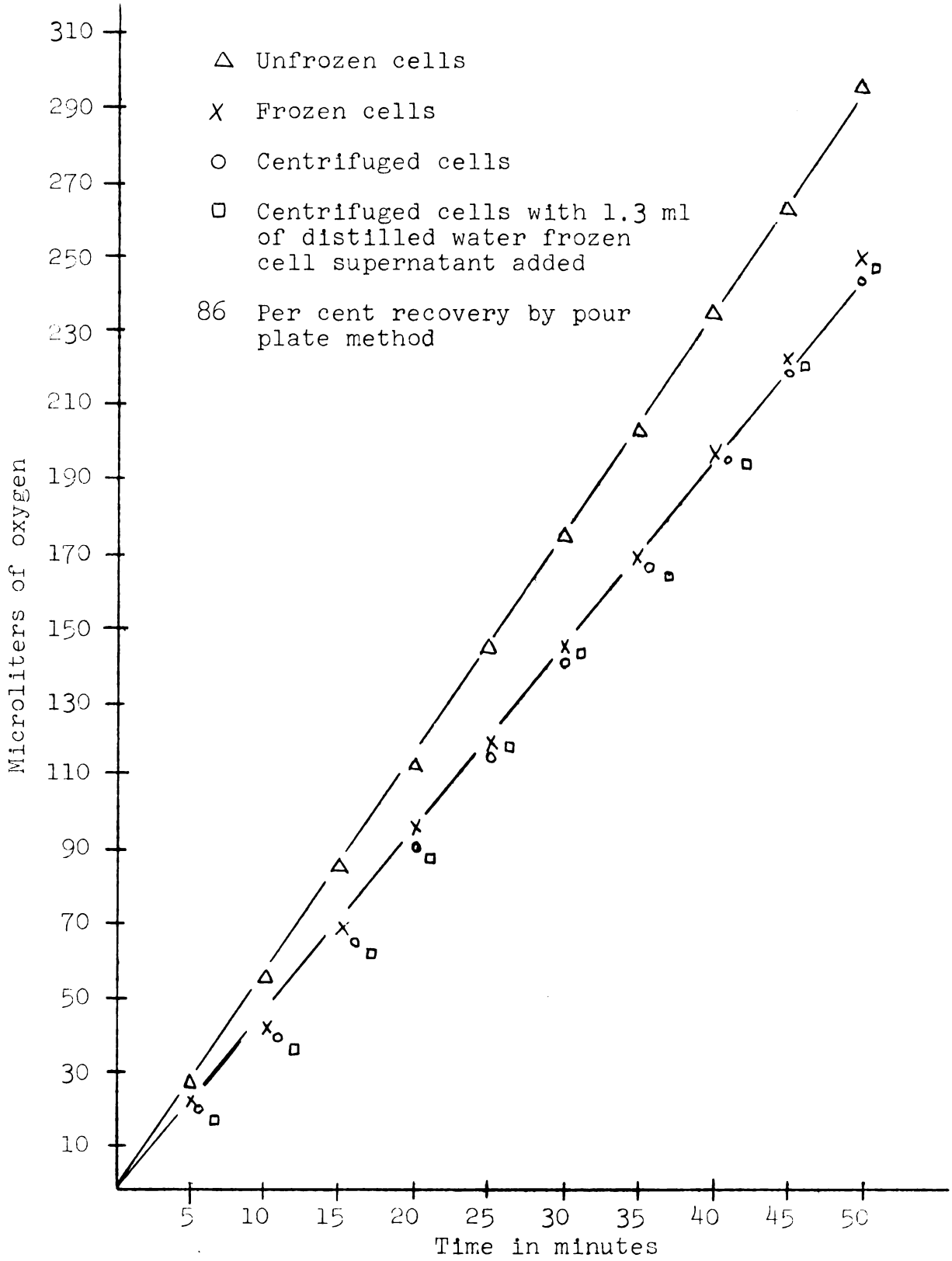


Fig. 5. Oxygen Uptake of Escherichia coli Cells Frozen in Distilled water

Two subsequent trials showed no deviation from that found in Figure 5.

Effect of adding 0.15 phosphate buffer and distilled water frozen cell supernatants to saline centrifuged cells. Results obtained by the addition of frozen cell supernatants from 0.15 M phosphate buffer and distilled water, individually to saline centrifuged cells is shown in Figure 6.

The phosphate buffer supernatant caused as much stimulation of oxygen uptake as the saline frozen cell supernatant when added to saline centrifuged cells (Fig. 6). The supernatant from cells frozen in distilled water, however, did not cause as much stimulation in the respiration rate as did the saline and phosphate supernatants (Fig. 6).

Two other trials substantiated these findings with little deviation from the general trend found in figure 6.

Effect of adding 0.85 % saline and distilled water frozen cell supernatants to 0.15 M phosphate buffer centrifuged cells. When 0.85 % saline and distilled water frozen cell supernatants were added individually to 0.15 M phosphate buffer centrifuged cells (Fig 7), an increase in the respiration rate over that of the

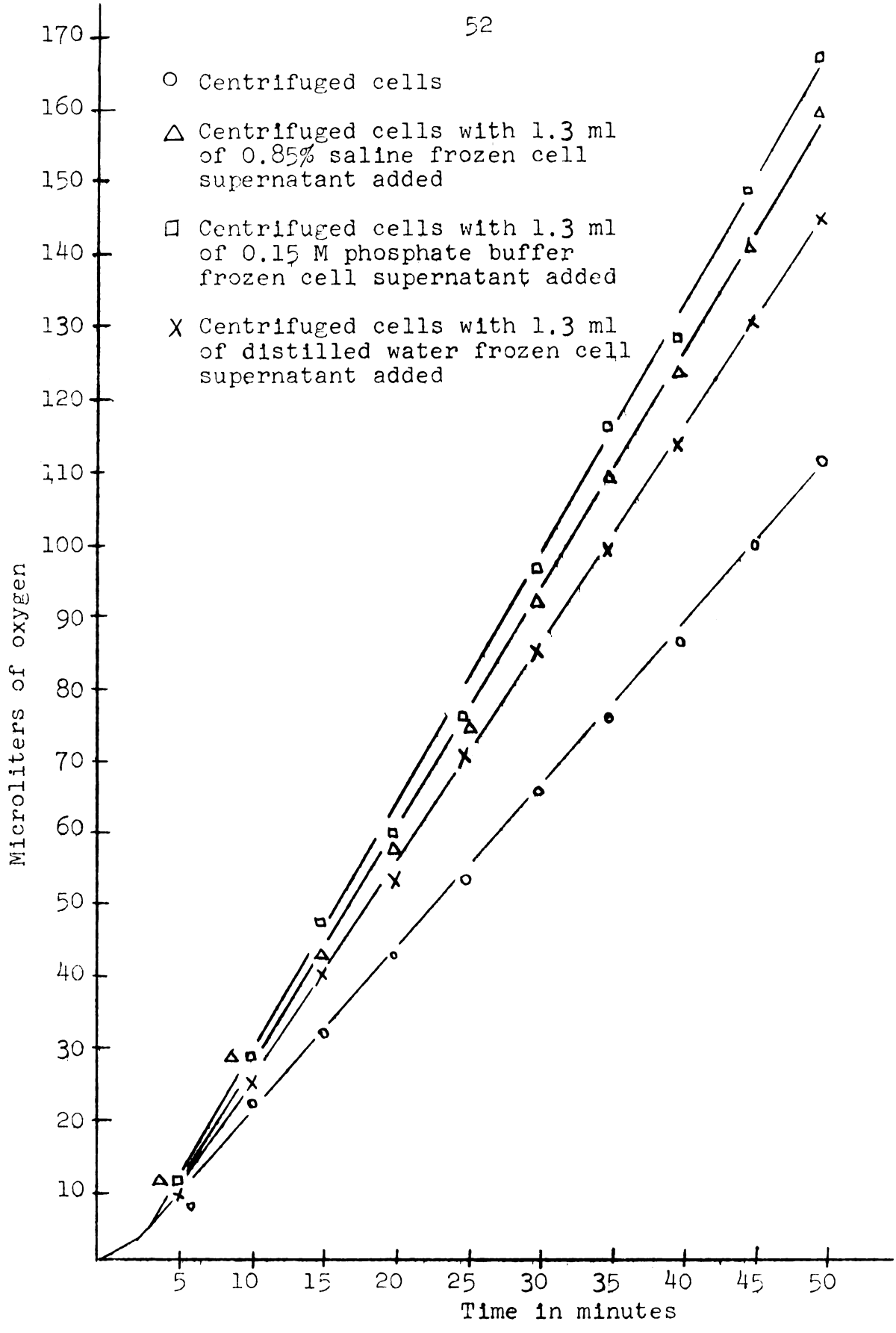


Fig. 6. Effects of Adding Supernatants From the Three Freezing Methods to Escherichia coli Centrifuged Cells Frozen in 0.85% Saline As Measured by Oxygen Uptake

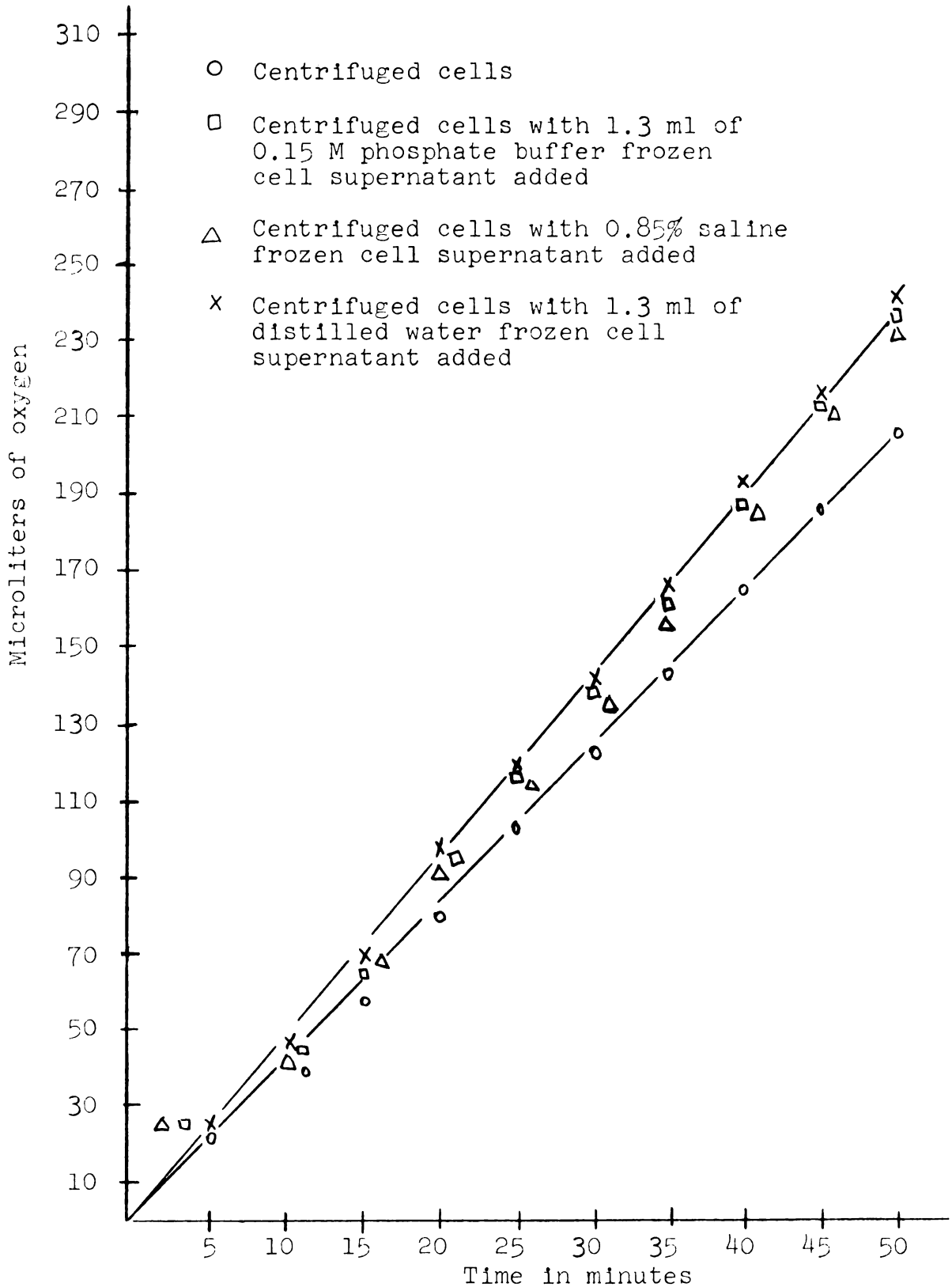


Fig. 7. Effects of Adding Supernatants From Three Freezing Methods to Escherichia coli Centrifuged Cells Frozen in 0.15 M Phosphate Buffer as Measured by Oxygen Uptake

centrifuged cells was noted. The addition of 0.15 M phosphate buffer frozen cell supernatant to the centrifuged cells gave an oxygen uptake of 230 ul, almost the same as the 0.85 % saline and distilled water supernatants (Figure 7). Subsequent trials gave data in agreement with that of Figure 7.

Effect of adding 0.85 % saline and 0.15 M phosphate buffer frozen cell supernatants to distilled water centrifuged cells. As shown previously, freezing caused the least amount of damage to cells suspended in distilled water. Their own frozen cell supernatant caused no increase in oxygen uptake over that of the centrifuged cells. Addition of 0.85 % saline and 0.15 M phosphate buffer frozen cell supernatant to the distilled water centrifuged cells, caused no stimulation but rather a decrease in oxygen uptake (Fig. 8). There also was no significant difference in oxygen uptake between the 0.85 % saline, and 0.15 M phosphate buffer frozen cell supernatants.

The decrease in oxygen uptake due to the presence of 0.85 % saline and 0.15 M phosphate supernatants, can possibly be accounted for on the basis of the endogenous respiration rate. The endogenous respiration rate for the distilled water centrifuged cells

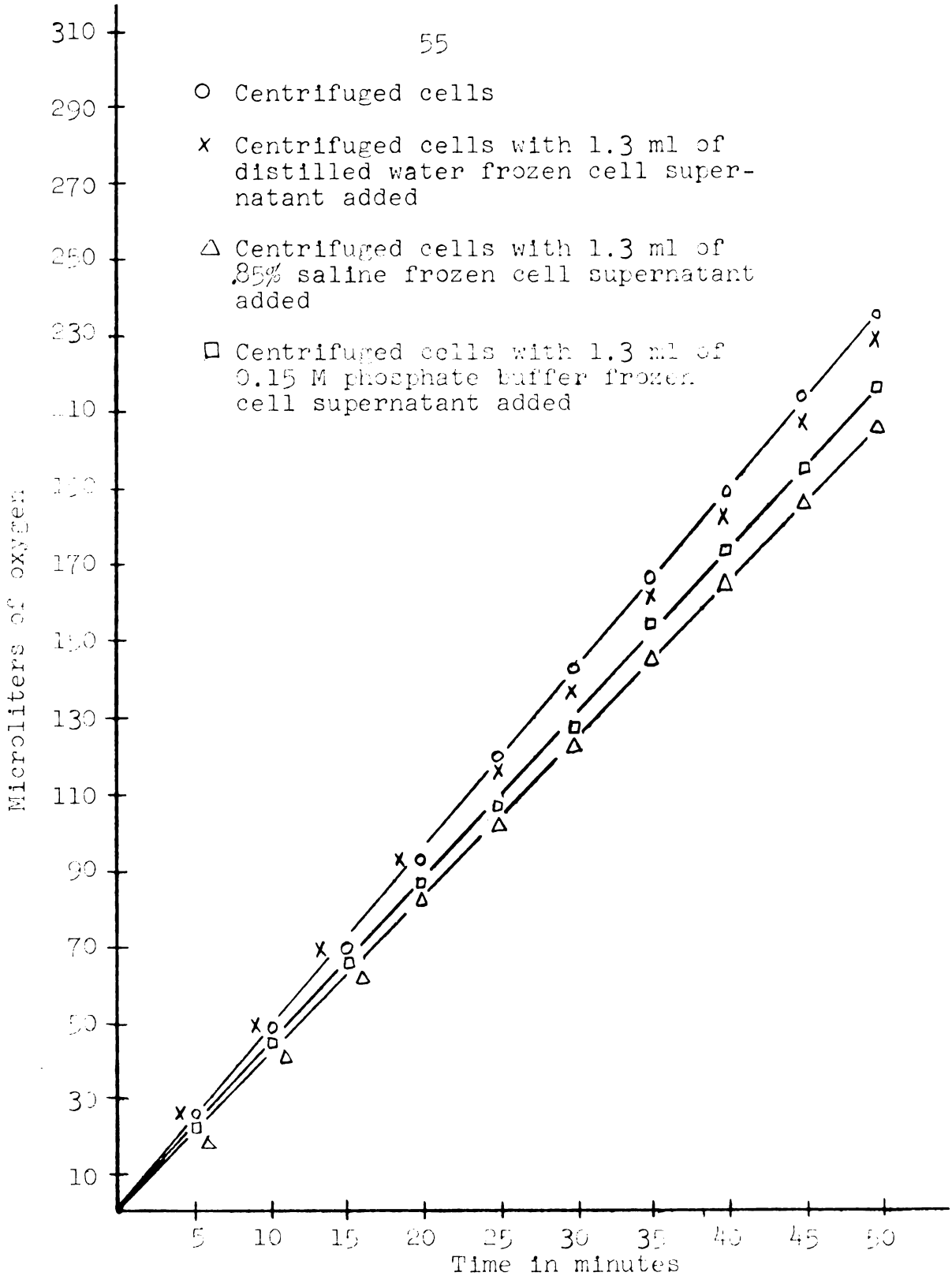


Fig. 8. Effects of Adding Supernatants From Three Freezing Methods to Escherichia coli Centrifuged Cells Frozen in Distilled Water As Measured by Oxygen Uptake

was 39 ul of oxygen, whereas it was increased to 56 and 51 ul when the saline and phosphate supernatants were added respectively (Table 1).

Effect of freezing on the endogenous rates. The saline frozen cell supernatant also seemed to cause an increase in the endogenous respiration rate of 0.15 M phosphate centrifuged cells greater than their own supernatant (Table 1). The distilled water and phosphate buffer frozen cell supernatants did not increase the endogenous rate of saline centrifuged cells over that of their own supernatant (Table 1).

The endogenous respiration rates of saline and 0.15 M phosphate centrifuged cells can be increased by the addition of their respective frozen cell supernatants. The addition of 1.3 ml of the supernatants in both cases, gave the greatest increase (Table 2). Distilled water and 0.06 M phosphate buffer frozen cells, did not exhibit this response.

Table 3 shows that the endogenous respiration rates were in most cases, at least doubled after freezing in the various suspending agents. If the frozen cells were washed once after removing their supernatant, no deviation was noted (Table 3, trial 2).

Effect of 0.85 % saline frozen cell supernatant on

Table 1. The effect on the endogenous respiration rate of Escherichia coli centrifuged cells frozen in three diluents by the addition of the three frozen cell supernatants as measured by oxygen uptake.

Centrifuged cells frozen in:	Frozen Cell Supernatant		
	0.85% Saline	distilled water	0.15 M phosphate buffer
0.15 M phosphate buffer	56*	40	44
0.85% Saline	53	55	55
Distilled water	56	35	51

* Microliters of oxygen consumed in 50 minutes

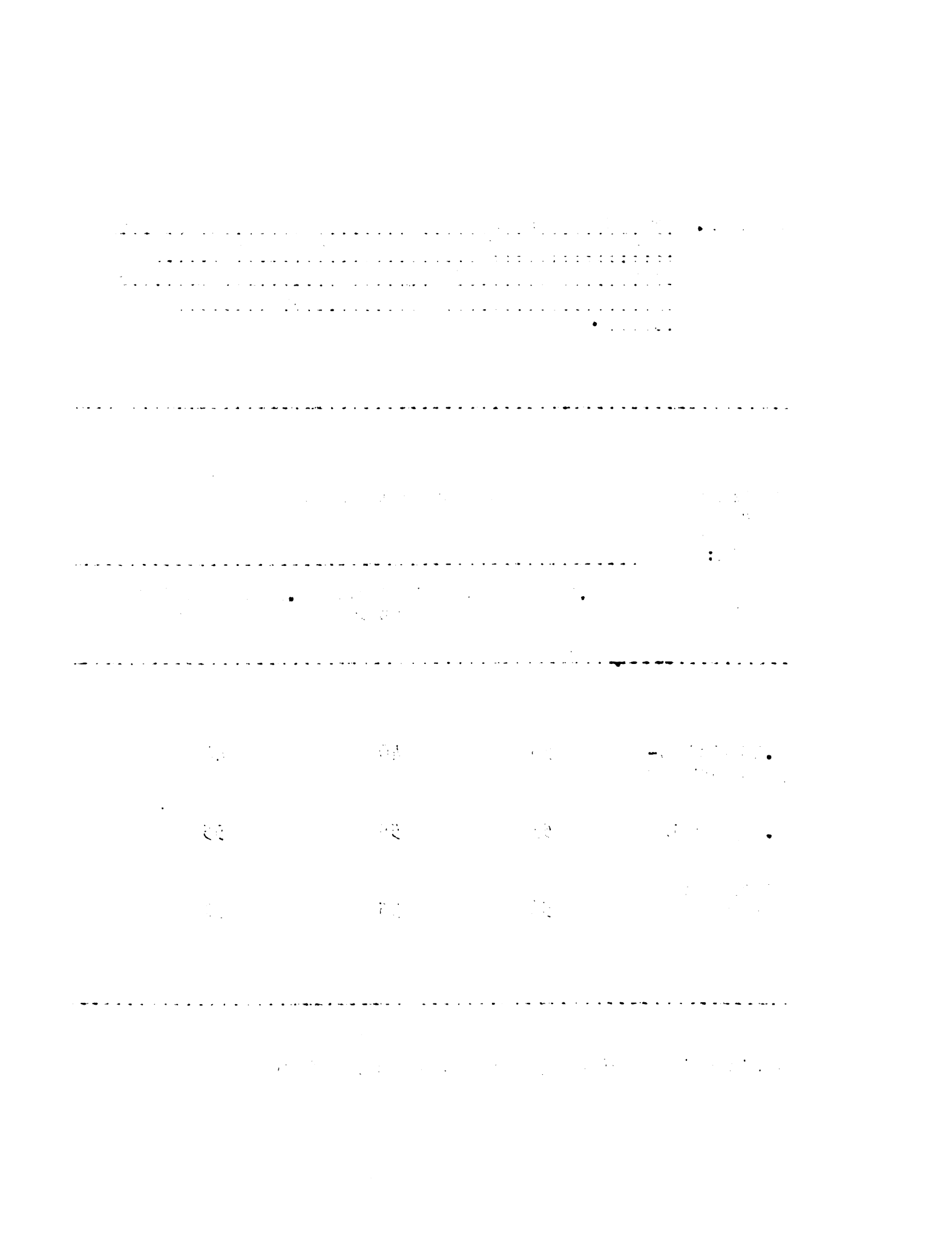


Table 2. Endogenous respiration rate of Escherichia coli cells , frozen in four suspending agents as measured by oxygen uptake.

Cell population	Suspending Agent			
	distilled water	0.85% Saline	0.15 M phosphate buffer	0.06 M phosphate buffer
Centrifuged cells	35*	31	31	35
Frozen cells	34	43	38	34
Centrifuged cells with 1.3 ml of frozen cell supernatant	33	51	44	32

* Microliters of oxygen consumed in 50 minutes

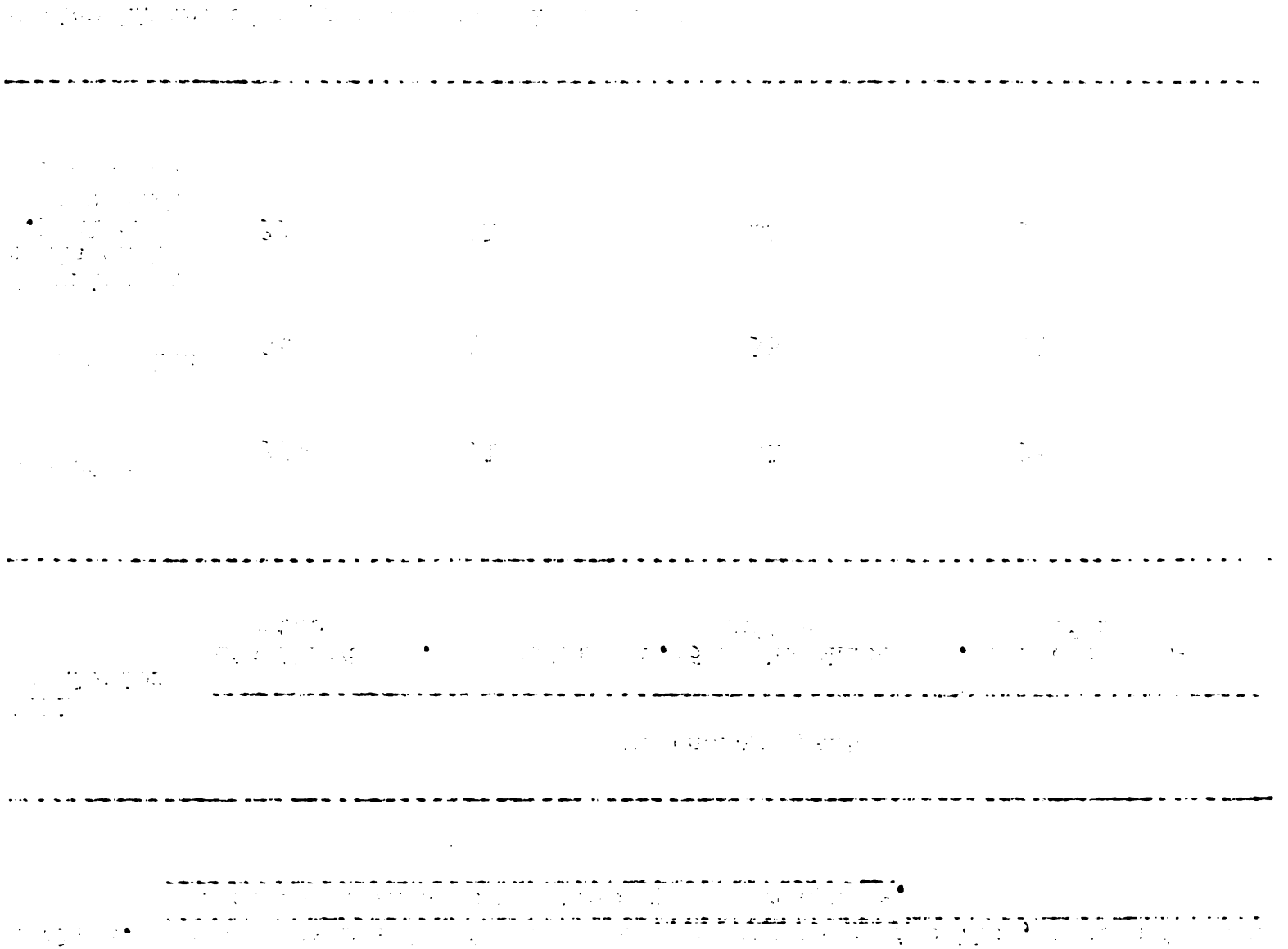


Table 3. Endogenous respiration rates for Escherichia coli cells, suspended in different agents before and after freezing, as measured by oxygen uptake.

Suspending diluent	Oxygen uptake in microliters in 50 minutes					
	Before freezing			After freezing		
	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2**	Trial 3
0.85% saline	19	21	18	45	35	30
0.06 M PO_4 buffer	12	13	16	31	35	19
0.15 M PO_4 buffer	15	12	16	56	28	38
distilled water	15	16	13	35	41	43

* Frozen cell supernatant removed (centrifuged cells)
**Centrifuged cells washed once

centrifuged unfrozen cells. The results obtained when saline frozen cell supernatant was added to unfrozen centrifuged cells is shown in Figure 9. The unfrozen cells without supernatant had an oxygen uptake of 285 ul. When 1.3 ml of the frozen cell supernatant was added, the oxygen uptake was only 275 ul. The endogenous rate for the cells with the supernatant was 29 ul as opposed to 13 ul for cells without supernatant. Similar results were obtained in two other trials.

Characterization of the 0.85 % saline frozen cell supernatant from frozen E. coli cells. Upon dialysis for 24 hours, the saline frozen cell supernatant failed to increase the oxygen uptake of saline centrifuged cells (Fig. 10). The oxygen uptake for the centrifuged cells with 1.3 ml of the saline frozen cell supernatant added, was 158 ul, as compared to 123 ul for the centrifuged cells and 124 ul for centrifuged cells with 1.3 ml of dialyzed supernatant added.

Heating the saline frozen cell supernatant for 30 minutes at 100 C did not inactivate the stimulatory property or properties of the supernatant (Fig 11). The oxygen uptake of the saline centrifuged cells for 50 minutes was 60 ul as compared to 150 and 145 ul for the heated and unheated supernatants respectively for the

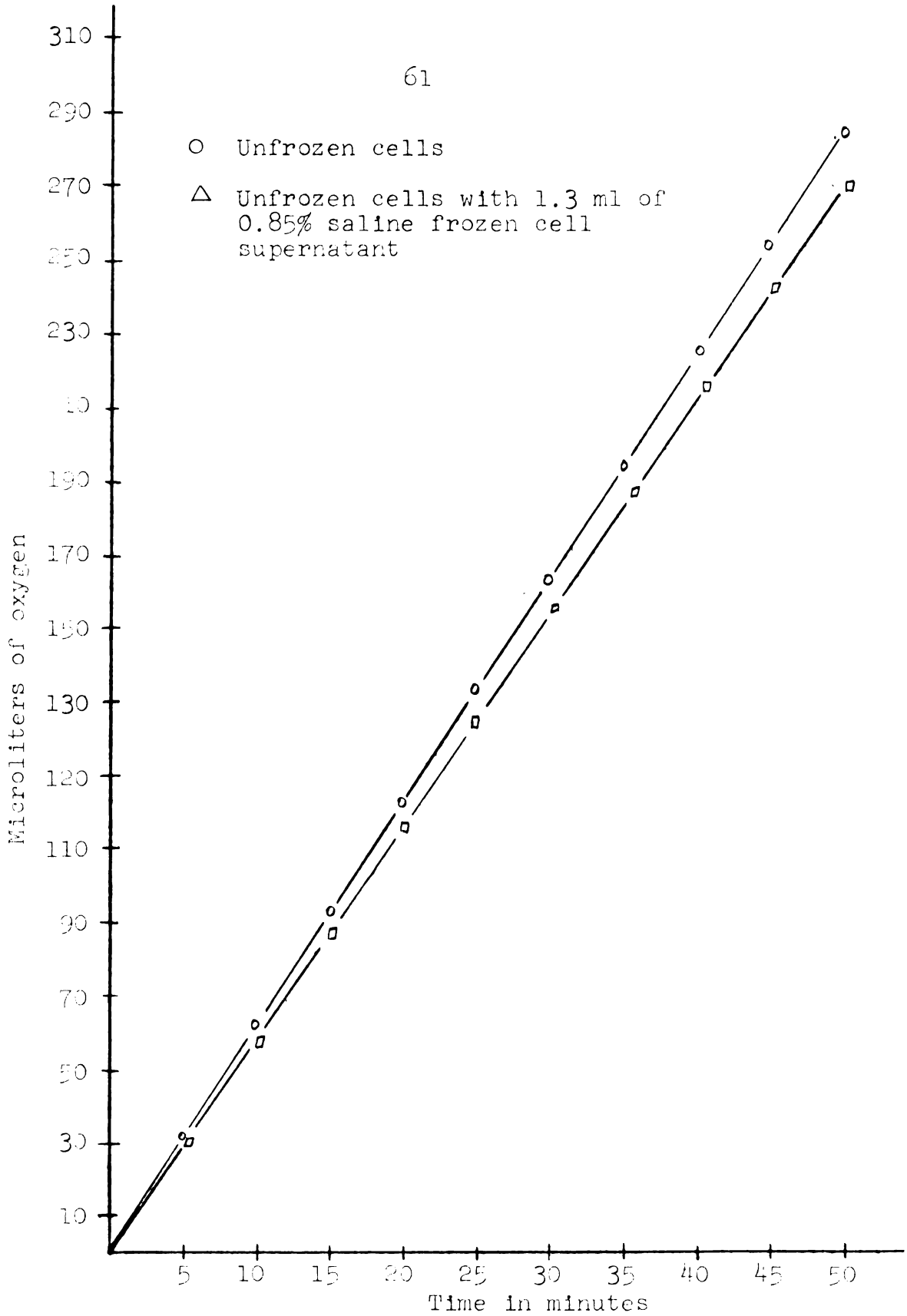


Fig. 9. Effect of Adding Saline Frozen Cell Supernatant to Unfrozen Centrifuged Cells of Escherichia coli Suspended in 0.85% Saline As Measured by Oxygen Uptake

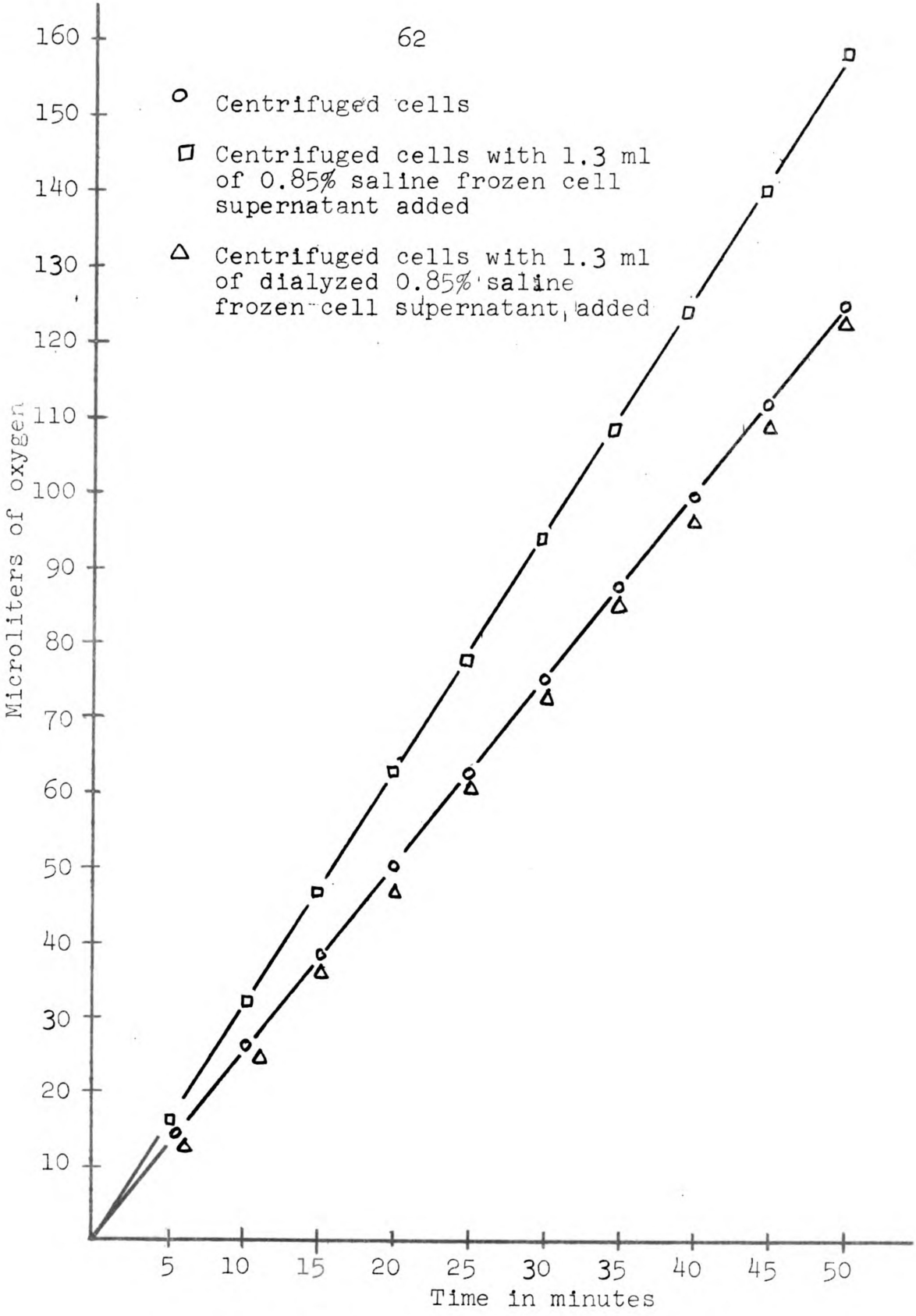


Fig. 10. Effect of Adding Dialyzed Saline Frozen Cell Supernatant to Escherichia Coli Centrifuged Cells Frozen in 0.85% Saline As Measured By Oxygen Uptake

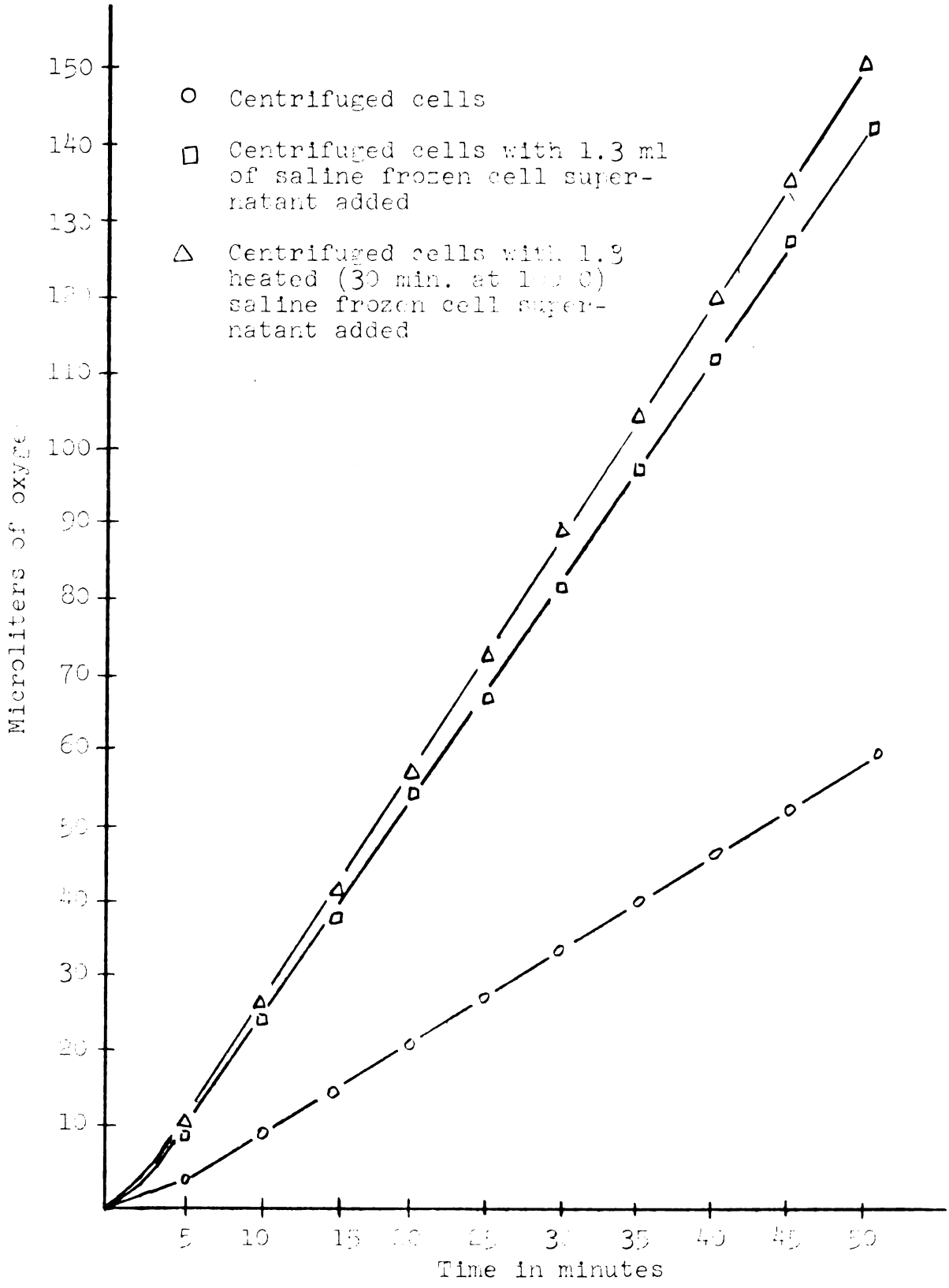


Fig. 11. Effect of Adding Heated Saline Frozen Cell Supernatant to Escherichia coli Centrifuged Cells Frozen in 0.85% Saline As Measured by Oxygen Uptake

same period of time.

The absorption spectrum of the saline frozen cell supernatant showed a strong maximum absorption at 260 mu.

Norit A.,an activated charcoal, was used to determine whether or not the activity of the saline frozen cell supernatant could be removed by adsorption. The centrifuged cells with 1.3 ml of supernatant, exhibited an oxygen uptake of 148 ul in 50 minutes, whereas centrifuged cells alone, had an uptake of only 93 ul (Fig. 12). After treatment of the supernatant with Norit A, 1.3 ml of the supernatant was added to the centrifuged cells. A decrease in uptake from 148 ul to 105 ul was noted.

It has been suggested (Halvorson et al., 1961) that water soluble cofactors might be leaching out of the frozen cells, causing injury and death. To ascertain whether or not this was happening in our freezing system, certain cofactors were added to the saline centrifuged cells to determine if they could replace the activity of the saline frozen cell supernatant.

Figure 13 and Table 4 show the results obtained with ATP, ADP, and $MgCl_2$. Table 4, which contains the same data as Figure 13, is presented for the purpose of clarity. ADP did not increase the oxygen uptake of the

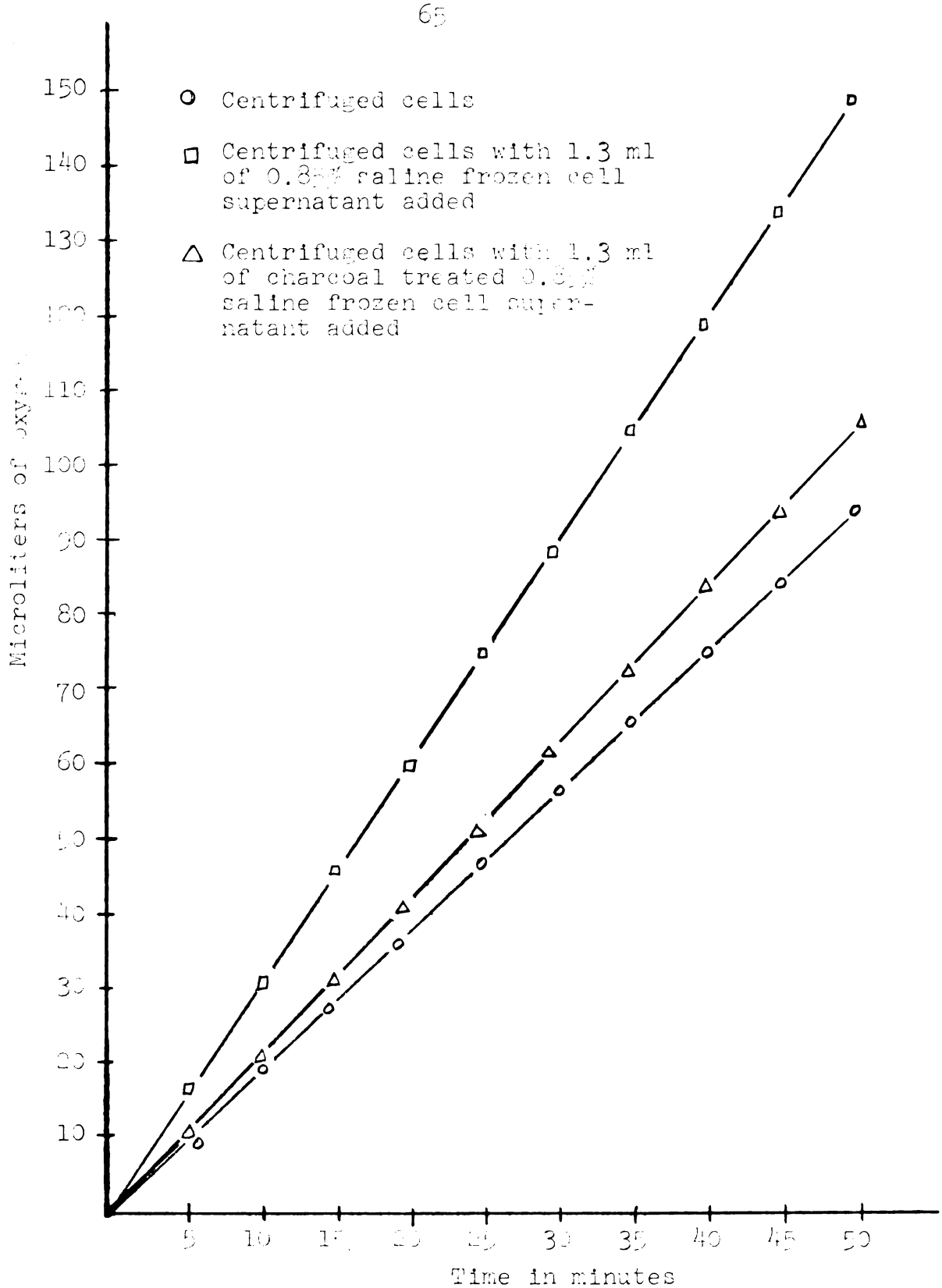


Fig. 12. Effect of Adding Charcoal Treated 0.85% Saline Frozen Cell Supernatant to *Escherichia coli* Centrifuged Cells Frozen in 0.85% Saline As Measured by Oxygen Uptake

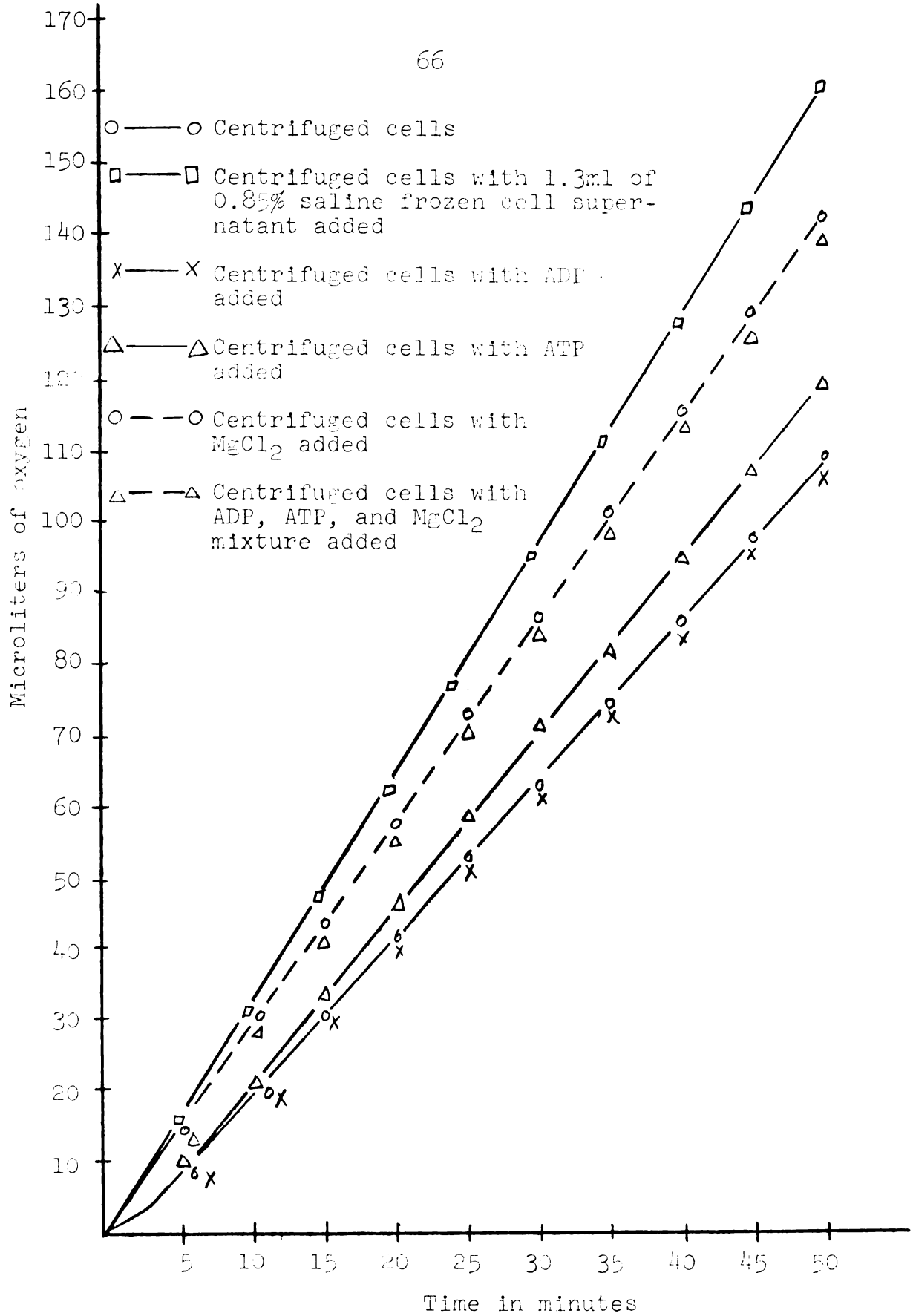


Fig. 13. Effect of Adding ADP, ATP, and $MgCl_2$ to *Escherichia coli* Centrifuged Cells Frozen in 0.85% Saline As Measured by Oxygen Uptake

Table 4. Effect of Adding ADP, ATP, and $MgCl_2$ to Escherichia coli centrifuged cells, frozen in 0.85% Saline, as measured by oxygen uptake.

Cofactor	Microliters of oxygen consumed in 50 minutes
ADP	107
ATP	120
$MgCl_2$	143
ADP, ATP, $MgCl_2$	139
Centrifuged cells	110
Centrifuged cells with 1.3 ml of saline frozen cell supernatant added	162

centrifuged cells and ATP increased it only 10 ul. However, $MgCl_2$ and the ADP, ATP, $MgCl_2$ mixture, increased the oxygen uptake of the centrifuged cells about 40 ul. The $MgCl_2$ and the mixture therefore could replace about half of the activity of the saline frozen cell supernatant. As would be expected, ADP, ATP, and $MgCl_2$ had no effect on unfrozen cells.

Two micromoles of NAD were also added to the centrifuged cells. The saline frozen cell supernatant, when added to centrifuged cells, caused a 50 ul increase in oxygen uptake, whereas the NAD increased the oxygen uptake 44 ul, when added to centrifuged cells (Fig. 14).

The assay for NAD in the saline frozen cell supernatant with glucose dehydrogenase is shown in figure 15. When the NAD was replaced by the saline frozen cell supernatant in the presence of glucose and the enzyme, no change in optical density was noted (Fig. 15).

Using citrate as substrate with unfrozen organisms, no oxygen uptake was noted as would be expected. Neither was citrate metabolized by frozen organisms. The oxygen uptake with citrate as substrate for the centrifuged cells, was the same as the endogenous (Table 5). The addition of acetate along with the citrate, showed no greater uptake than with acetate alone (Table 5).

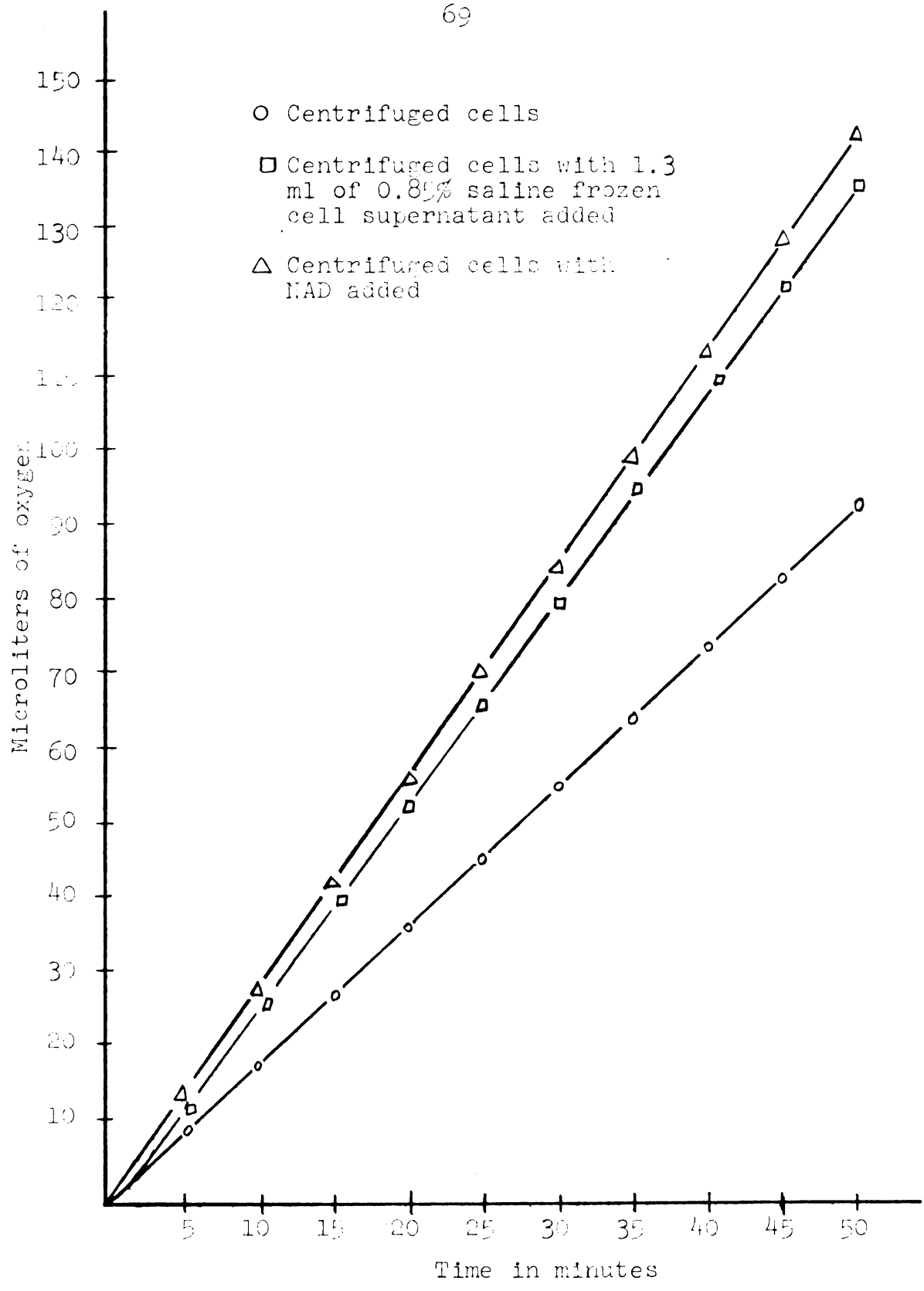


Fig. 14. Effect of Adding NAD to Escherichia coli Centrifuged Cells Frozen In 0.85% Saline As Measured by Oxygen Uptake

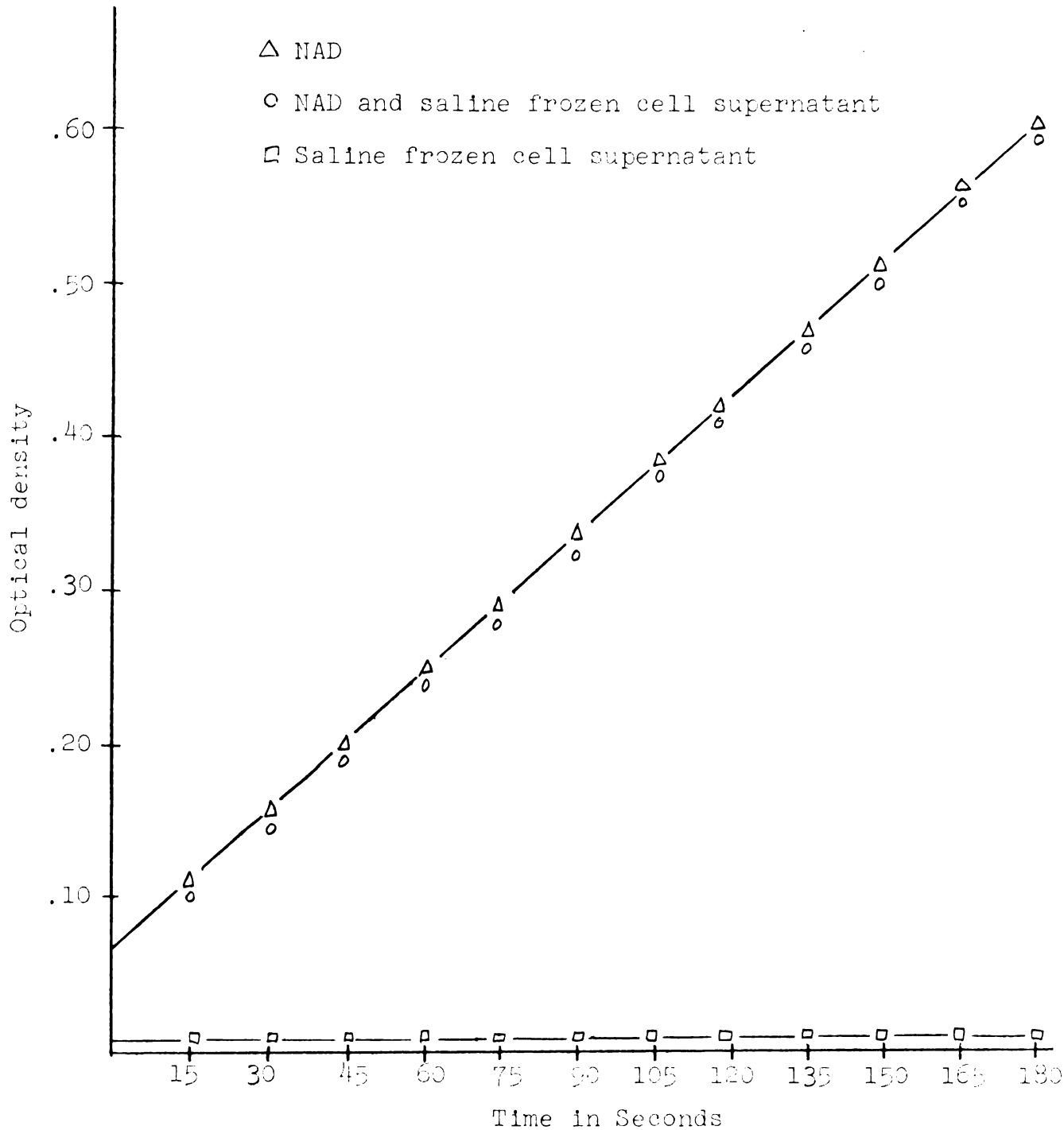
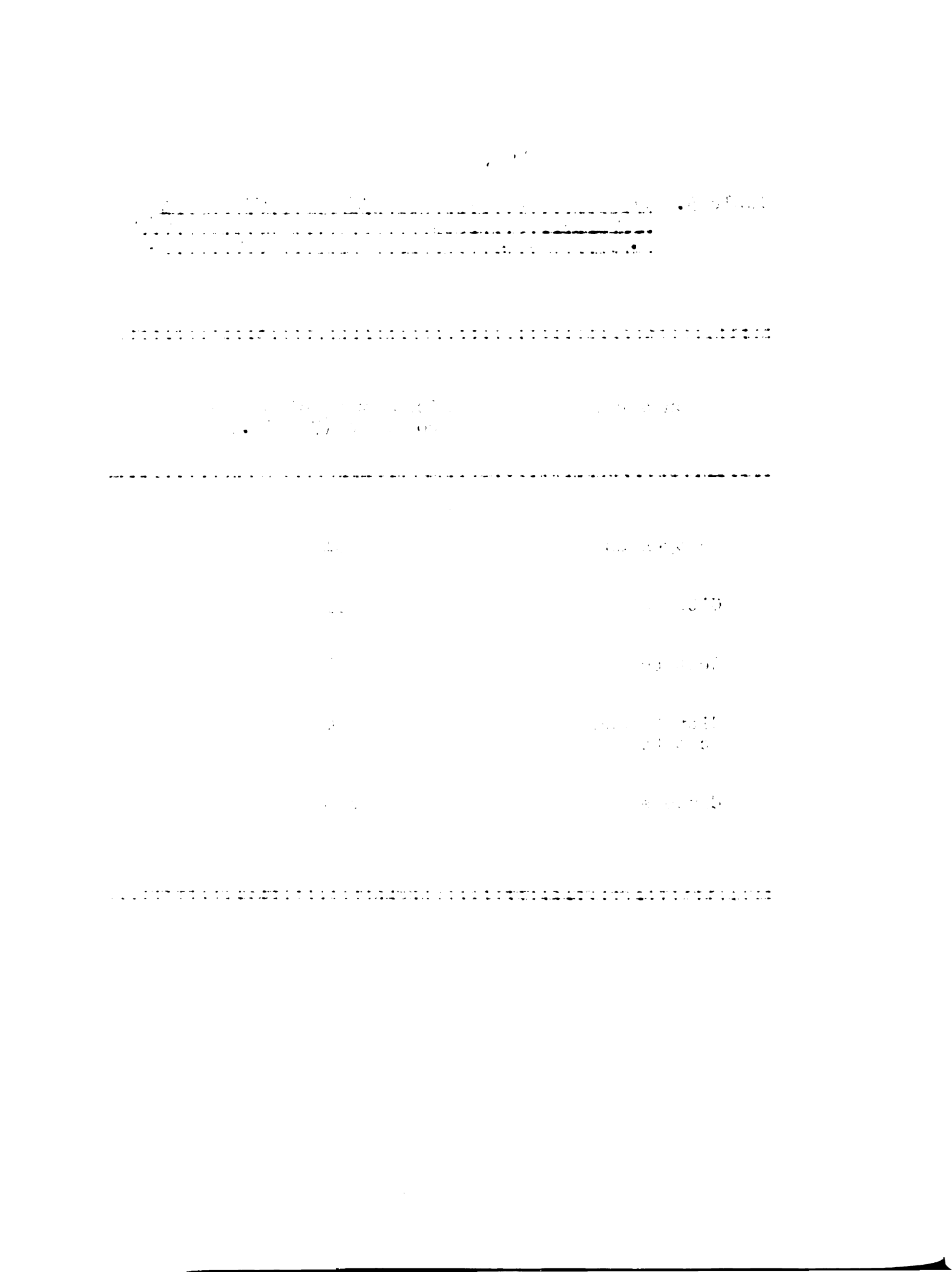


Fig. 15. Assay For NAD In the Saline Frozen Cell Supernatant With Glucose Dehydrogenase

Table 5. Permeability and utilization of citrate by Escherichia coli centrifuged cells frozen in 0.85% Saline, as measured by oxygen uptake.

Substrate	Microliters of oxygen consumed (70 min.)
Endogenous	51
Citrate	53
Acetate	83
Citrate and Acetate	86
Glucose	100



Citrate in the presence of acetate was not metabolized by unfrozen cells.

DISCUSSION

Comparison of the four freeze suspending agents showed that distilled water gave the best per cent of viable cells recovered followed then by 0.15 M phosphate buffer, 0.06 M phosphate buffer and finally 0.85 % saline. These results are in general agreement with those found in the literature.

The per cent recoveries obtained with 0.06 M and 0.15 M phosphate buffer suspending media, were not those expected. It was expected that 0.15 M phosphate buffer would be as damaging, if not more so, than 0.06 M phosphate buffer. But cells frozen in 0.06 M phosphate buffer suffered almost twice as much damage as those frozen in 0.15 M phosphate buffer. It does not seem that ionic strength of the buffers was an important factor. It is possible that the higher molarity of the 0.15 M phosphate buffer in some manner, protects the cells during the freezing and thawing process.

In general, it seems that the effect of freezing in different diluents is dependent on the electrolytes used and not upon the molarity or ionic strength of the solutions.

Decreases in oxygen uptake were found after freezing in each of the four diluents, with 0.85 % saline showing the greatest decrease. The decreases in uptake after freezing in 0.06 and 0.15 M phosphate buffers and distilled water were of about the same magnitude. There seemed to be no correlation between per cent recovery and decrease in oxygen uptake after freezing for the four diluents. This is illustrated by a comparison of 0.06 M phosphate buffer and distilled water results. Thus, it is also shown that the oxygen uptake is influenced more by the electrolyte used than by the ionic strength.

Removal of the frozen cell supernatant from cells frozen in 0.85 % saline and 0.06 M phosphate buffer, caused the greatest decrease in oxygen uptake. On the other hand, only about one-half as much of a decrease was noted when the frozen cell supernatant was removed from 0.15 M phosphate buffer frozen cells. No decrease was found for distilled water frozen cells.

If the frozen cell supernatants were added to their respective centrifuged cells, the oxygen uptake could be increased to the level that it was before removal.

The only exception to this was with cells frozen in 0.85 % saline. Addition of 1.3 ml of the saline

frozen cell supernatant to their centrifuged cells, in most instances, increased the oxygen uptake over that of the frozen cells. With 0.06 and 0.15 M phosphate buffers centrifuged cells, addition of 1.3 ml of their respective frozen cell supernatants raised the oxygen uptake to the level of the frozen cells, but no higher.

The difference between the action of the saline frozen cell supernatant and that of the phosphate buffers, might be due to the increased need for the active material (s) in the saline supernatant by the saline centrifuged cells. This is very possible since saline frozen cells sustained the greatest injury and damage.

The question now arises as to the explanation of this stimulation of the centrifuged cells by the frozen cell supernatants. Three possibilities seem likely. They are: leakage of cellular material, disruption of the cells, or a combination of both.

If cell disruption were the major cause of the stimulation, one would expect to obtain a cell free preparation. In the case of saline, an 80 to 90 % cell free preparation could be obtained. Assuming no loss of cofactors, the frozen cell supernatants

then would be expected to metabolize glucose. This did not occur. However, this does not eliminate the possibility of some cell lysis--perhaps at a very low level. But Gritsavage (1963) was unable to detect any amino acids or deoxyribonucleic acid in various frozen cell supernatants. However, if the cells were disrupted using ultra-sonic vibration, deoxyribonucleic acid could be detected.

The other possibility is cell leakage. Hartsell (1961), Lund (1961), and Smith (1954) advocate this theory. During the freezing and thawing process, cellular materials leach from the cells. This causes a metabolic injury to the cells which could be overcome by re-entry of material from the frozen cell supernatants. Our results seem to support this occurence. Further, the results demonstrate a correlation between freeze damage in terms of per cent viability and leakage--the greater the freeze damage, the greater the leakage.

Addition of distilled water frozen cell supernatant to saline centrifuged cells, demonstrated that leakage was occurring when cells were frozen in distilled water. However, the activity of the distilled water supernatant was only two-thirds as great as that

of the saline supernatant, indicating that the leakage was not as great.

The 0.15 M phosphate supernatant gave the same amount of activity as did the saline supernatant when added to saline centrifuged cells. It is therefore probable that the leakage from the phosphate frozen cells is sufficient to supply the needs of the saline centrifuged cells.

The saline and distilled water frozen cell supernatants, when added to 0.15 M phosphate buffer centrifuged cells, gave the same results as the phosphate supernatant. This again demonstrates that the distilled water frozen cell supernatant contains an active substance or substances which can cause stimulation. The saline frozen cell supernatant was also able to stimulate the phosphate centrifuged cells to the same degree as was their own supernatant.

Results with distilled water centrifuged cells and the three frozen cell supernatants showed that 0.15 M phosphate and saline frozen cell supernatants cannot stimulate the distilled water centrifuged cells. In fact they seemed to decrease the activity. However, it was found that the endogenous respiration rates of the distilled water centrifuged cells were significantly

increased when either the saline or phosphate frozen cell supernatants were added. It is possible that these supernatants contain a utilizable substrate and the increase in the endogenous rate is due to this. When the endogenous values are substracted from the exogenous values for the saline and phosphate supernatants, an error is introduced.

These results demonstrated that the leakage materials were similar in the sense that they could stimulate cells frozen in other diluents with the exception of distilled water centrifuged cells.

The reason that the distilled water centrifuged cells were not stimulated by any of the supernatants probably can be explained on the basis of cell viability after freezing. Since between 80 to 90 % of the cells can be recovered, the percentage of injured cells would be small. These cells are probably utilizing the material in the supernatants but to such a small extent that it cannot be detected with the methods used.

Hartsell (1961) has postulated that his leakage material is a result of a cellular build-up of an intermediate compound in a sequence of reactions. In other words, product A goes to product B and this product combined into C, but the use of C in D cannot occur

until the cells are defrosted. Thus, product C accumulates as the cells are held in storage. Our data suggests that this might be occurring.

The endogenous respiration rates for cells frozen in all the suspending agents were always greater after freezing had occurred. The results further showed that the endogenous respiration rates of saline and 0.15 M phosphate buffer centrifuged cells were increased when their respective frozen cell supernatants were added. This, however, did not seem to be true for cells frozen in 0.06 M phosphate buffer and distilled water. Also, distilled water and 0.15 M phosphate buffer supernatants could increase the endogenous rate of saline centrifuged cells to the same level as the saline supernatant. This was true for the addition of saline and distilled water supernatants to phosphate centrifuged cells, except the saline supernatant increased the uptake more than phosphate or distilled water did.

One could postulate from these data, that there is a build up of some intermediate or intermediates in a sequence of biochemical events. The cells once thawed, could begin to metabolize this intermediate or intermediates, and thus account for the increased endogenous respiration rates after freezing and thawing.

Washing the centrifuged cells once did not lower the rate, therefore it would seem that the material is intracellular. By addition of the frozen cell supernatants, more substrate is added and thus an even higher rate of oxygen uptake or the supernatant is actually stimulating the endogenous rate. Further study would be necessary to establish such sequence of events.

Unfrozen cells suspended in saline, were not stimulated by the saline frozen cell supernatant. The endogenous rate of these unfrozen cells was increased by addition of the saline frozen cell supernatant, indicating again, the possibility of a utilizable substrate being present in low concentrations. Two possible reasons why unfrozen cells were not stimulated by the supernatant, could be either they have no need for the substance or substances in the supernatant, or that these substances might be impermeable.

The stimulatory factor or factors, can be removed from the saline frozen cell supernatant by dialysis. The stimulatory effect, therefore seems to reside in low molecular weight compounds.

Activity of the saline frozen cell supernatant was not lost or decreased, due to heating for 30 minutes at 100 C. Under these conditions, it is heat

stable.

Treatment of the cells with Norit A, removed about two-thirds of the stimulatory activity of the saline frozen cell supernatant. Perhaps a longer treatment of the supernatant or incubation at a temperature higher than 37 C would have removed more of the activity.

Since the saline frozen cell supernatant had a maximum absorption at 260 um, it was thought that ATP or ADP could possibly be the active components.

ATP and ADP in the amounts added, were not able to simulate the activity of the frozen cell supernatant. $MgCl_2$, on the other hand, was able to increase the oxygen uptake about one-half that of the saline frozen cell supernatant. Adding a mixture of ATP, ADP, and $MgCl_2$ to the saline centrifuged cells gave almost the same results as $MgCl_2$ alone. This was probably due to the $MgCl_2$.

No effort was made to assay for the presence of Mg^{++} in the frozen cell supernatant, but it is possible that during freezing and thawing of the cells, Mg^{++} could be lost from the cell, perhaps due to dehydration of the cell or an unfavorable concentration gradient, surrounding the cell.

Bovarnick (1954) and Bovarnick and Allen (1957)

have demonstrated with typhus rickettsiae, that nicotinamide adenine dinucleotide can restore respiratory activity after freezing and thawing of this organism. The presence of NAD was also detected in the frozen cell supernatant.

Addition of NAD to saline centrifuged cells, did replace the activity of the saline frozen cell supernatant. To explain the action of NAD stimulation, it must be assumed that NAD can pass through the cell membrane in order to cause the stimulation. E. coli, when not frozen, is impermeable to NAD. It is therefore possible that during freezing and thawing with our system, there arises an altered permeability of the cell membrane.

Glucose dehydrogenase was used to assay for NAD in the saline frozen cell supernatant. No NAD could be detected. It was also determined that the frozen cell supernatant did not contain inhibitors which might have interferred with the action of the glucose dehydrogenase.

Efforts were also made to determine if citrate, which was impermeable to our strain of E. coli, could be metabolized after freezing. It was found that it could not be metabolized, whether alone or in the presence of acetate.

SUMMARY

It was found that the number of viable cells surviving freezing and thawing for 22 hours at -16 C was dependent on the suspending menstrum. The best survival was obtained with distilled water, followed by 0.15 M phosphate buffer, 0.06 M phosphate buffer, and 0.85 % saline.

Decreases in oxygen uptake after freezing, were noted in cells suspended in all the suspending agents. Cells frozen in 0.85 % saline had the greatest decrease, while cells frozen in the phosphate buffers and distilled water showed similar smaller decreases.

Removal of the frozen cell supernatants demonstrated that the oxygen uptake could be further decreased, with the exception of distilled water suspended cells. By re-introducing the supernatant to the centrifuged cells, the oxygen uptake could be restored. In the case of cells frozen in 0.85 % saline, the level of oxygen uptake could be increased over that of the frozen cells. Evidence indicated that cell leakage had occurred during freezing and thawing with resulting cell injury. There also seemed to be a correlation between number of viable organisms sur-

viving freezing, and cellular leakage.

Testing of 0.85 % saline, 0.15 M phosphate buffer, and distilled water frozen cell supernatants against each of the opposite centrifuged cells revealed (1) that cellular leakage did occur when cells were frozen in distilled water but in low concentration, (2) distilled water centrifuged cell could not be stimulated, (3) there seemed to be a similarity between the frozen cell supernatants in regard to the active components.

The endogenous respiration rates were increased after freezing cells in the four suspending agents. Washing the cells once by centrifugation did not remove this increase.

The addition of the various frozen cell supernatants to centrifuged cells, caused a further increase in endogenous respiration.

Exceptions to this were cells frozen in 0.06 M phosphate and distilled water. However, the endogenous respiration of cells frozen in distilled water could be increased by the addition of 0.15 M phosphate buffer and 0.85 % saline frozen cell supernatants.

The active components of the saline frozen cell supernatant was found to be dialyzable, heat stable for 30 minutes at 100 C, and removable by adsorption to

Norit A.

The saline frozen cell supernatant had a maximum absorption spectrum at 260 um.

Nicotinamide adenine dinucleotide replaced the activity of 0.85 % saline frozen cell supernatant when added to the saline centrifuged cells. An assay for NAD, using glucose dehydrogenase, did not affirm its presence. The results with NAD indicated that a possible cellular permeability change might have taken place after freezing and thawing.

Adenosine diphosphate and adenosine triphosphate did not replace the activity of the saline frozen cell supernatant when added to saline centrifuged cells. $MgCl_2$ and a mixture of $MgCl_2$, ADP, and ATP replaced about one-half of the activity.

Citrate was not metabolized by cells frozen in 0.85 % saline.

Saline frozen cell supernatant was not able to increase the oxygen uptake of 0.85 % saline suspended unfrozen cells.

LITERATURE CITED

Ambrosini, R.A., and H.W. Bretz. 1963. Survival of Escherichia coli frozen in cell extracts. Bacteriol. Proc., p. 6.

Arpai, J. 1962. Nonlethal freezing injury to metabolism and motility of Pseudomonas fluorescens and Escherichia coli. J. Appl. Bacteriol. 10:297-301.

Arpai, J. 1963. Selective effect of freezing as reflected in growth curves. Folia Microbial. 8:18-26.

Baum, H.M. 1943. The resistance to low temperatures of a culture of Saccharomyces cerevisiae grown from a single cell. Biodynamica. 4:71-74.

Belehradek, J. 1935. Temperature and living matter. Protoplasm Monograph, Borntraeger, Berlin.

Bovarnick, M.R. 1954. Reversible inactivation of typhus rickettsiae. I. Inactivation by freezing. J. Gen. Physiol. 38:169-179.

Bovarnick, M.R., and E.G. Allen. 1957. Reversible inactivation of typhus rickettsiae at 0 C. J. Bacteriol. 73:56-62.

Bretz, H.W. 1958. Frozen storage and certain life processes of Escherichia coli. Ph.D. thesis, Purdue University, Lafayette, Ind.

Bretz, H.W. 1961. Death of Escherichia coli on cellophane and on membrane filters. Canad. J. Microbiol. 7:793-798.

Bretz, H.W., and K.B. Dasa. 1960. Protective substances for frozen Escherichia coli. Bacteriol. Proc., p. 38.

Bretz, H.W., and S.E. Hartsell. 1959. Quantitative evaluation of defrosted Escherichia coli. Food Res. 24:369-374.

Butterfield, C. T. 1932. The selection of a dilution water for bacteriological examinations. J. Bacteriol. 23:255-368.

Campbell, J.D. 1943. Resistance of yeast to low temperatures. Biodynamica. 4:65-70.

Chalmers, B. 1959. How water freezes. Sci. American 200:114-122.

Clement, M.T. 1961. Effects of freezing, freeze-drying, and storage in the freeze-dried and frozen state on viability of Escherichia coli cells. Canad. J. Microbiol. 7:99-106.

Currans, H.R., and F.R. Evans. 1937. The importance of enrichment in the cultivation of bacterial spores previously exposed to lethal agencies. J. Bacteriol. 34:179:189.

DeLamater, E.D., K.L. Babcock, and C.R. Mazzanti. 1959. On the leakage of cellular material from Bacillus megaterum. J. Bacteriol. 77:513-514.

Deotto, R. 1944. L'azione de freddo sulla respirazione della cellula bactterica. Arch. Exptl. Zellforsch. 25:101-105.

Dubos, R.J. 1937. Mechanism of the lysis of pneumococci by freezing and thawing, bile and other agents. J. Exptl. Med. 66:101-112.

Foulkes, E.C. 1954. Citrate metabolism and cell permeability. J. Bacteriol. 68:505.

Goetz, A., and S.S. Goetz. 1938. Vitrification and crystallization of photophyta at low temperatures. Proc. Am. Philos. Soc. 79:361-388.

Goresline, H.E. 1946. Microbiological examination of foods: Tentative methods for microbiological examination of frozen foods. Am. J. Pub. Health. 36:332-355.

Gritsavage, E.F. 1963. Personal communication.

Cunderson, M.F., and K.D. Rose. 1948. Survival of bacteria in a precooked, fresh frozen food. Food Res. 13:254-263.

Haines, R.B. 1938. The effect of freezing on bacteria. Proc. Roy. Soc. (London). 124B:451-463.

Halvorson, H.O., J. Wolf, and V.R. Srinivasan. 1961. Initiation of growth at low temperatures. Proc. Low Temperature Microbiology Sympos. Campbell Soup Co., Camden, N.J.

Harrison, A.P. 1956. Causes of death of bacteria in frozen suspensions. Antonie Van Leeuwenhoek J. Microbiol. Serol. 22:407-418.

Harrison, A.P. 1961. Aging and decline of bacteria in phosphate buffer. Bacteriol. Proc., p. 53.

Harrison, A.P., and R.E. Cerroni. 1956. Fallacy of "crushing death" in frozen bacterial suspensions. Proc. Soc. Exptl. Biol. Med. 91:577-579.

Hartman, P.A., and D.V. Huntsberger. 1961. Influence of subtle differences in plating procedure on bacterial counts of prepared frozen foods. J. Appl. Microbiol. 9:32-38.

Hartsell, S.E. 1951 a. The growth initiation of bacteria in defrosted eggs. Food Res. 16:96-106.

Hartsell, S.E. 1951 b. The longevity and behavior of pathogenic bacteria in frozen foods: the influence of plating media. Am. J. Pub. Health. 41:1072-1077.

Hartsell, S.E. 1959. Symposium on initiation of bacterial growth. Bacteriol. Rev. 23:250-253.

Hartsell, S.E. 1961. The microbiology of frozen foods. Proc. Low Temperature Microbiology Sympos. Campbell Soup Co., Camdem, N.J.

Hegarty, C.P., and O.B. Weeks. 1940. Sensitivity of Escherichia coli to cold-shock during the log growth phase. J. Bacteriol. 39:475-484.

Heinmets, F., W.W. Taylor, and J.J. Lehman. 1954 a. The use of metabolites in the restoration of viability of heat and chemically inactivated Escherichia coli. J. Bacteriol. 67:5-12.

Heinmets, F., W.W. Taylor, J.J. Lehman, and R.H. Kathan. 1954 b. The study of factors which influence metabolic reactivation of the ultraviolet inactivated Escherichia coli. J. Bacteriol. 67:511-522.

Higuchi, M., and T. Uemura. 1959. Release of nucleotides from yeast cells. Nature. 184:1381-1383.

Hilliard, C.M., C. Torossian, and R.P. Stone. 1915. Notes on the factors involved in the germicidal effects of freezing and low temperature. Science. 42:770-771.

Hilliard, C.M., and M.A. Davis. 1918. The germicidal action of freezing temperatures upon bacteria. J. Bacteriol. 3:423-431.

Holden, J.T. 1958. Degradation of intracellular nucleic acids and leakage of fragments by Lactobacillus arabinosus. Biochim. Biophy. Acta. 29:667-668.

Hollander, D.H., and E.E. Nell. 1954. Improved preservation of Treponema pallidum and other bacteria by freezing with glycerol. J. Appl. Microbiol. 2:164-170.

Howard, D.H. 1956. The preservation of bacteria by freezing in glycerol broth. J. Bacteriol. 71:625.

Jones, J.H., and F.W. Fabian. 1952. The viability of microorganisms isolated from fruits and vegetables when frozen in different suspending menstrua. Tech. Bull. Mich. Agric. Exp. Sta. 229:3-42.

Keith, S.C. 1913. Factors influencing the survival of bacteria at temperatures in the vicinity of the freezing point of water. Science. 37:877-879.

Kohn, A. 1960. Lysis of frozen and thawed cells of Escherichia coli by lyozyme and their conversion into speroplasts. J. Bacteriol. 79:697-706.

Kohn, A., and W. Szybalski. 1959. Lysozyme speroplasts from thawed Escherichia coli cells. Bacteriol. Proc., p. 126-127.

Lovelock, J.E. 1957. Denaturation of lipid-protin complexes as a cause of damage by freezing. Proc. Roy. Soc. (London). 147 B:427-433.

Lund, A.J. 1961. Personal communication (Cited from Halvoson, H.O., et al. 1961. Proc. Low Temperature Microbiology Sympos. Campbell Soup Co., Camden, N.J.

Luyet, B.I. 1961. Recent developments in cryobiology and their significance in the study of freezing and freeze-drying of bacteria. Proc. Low Temperature Microbiology Sympos. Campbell Soup Co., Camden, N.J.

Luyet, B.I., and P.M. Gehenio. 1940. Life and death at low temperatures. Biodynamica, Normandy, Mo.

Macfadyen, A. and S. Rowland. 1900. Further note on the influence of temperature of liquid air on bacteria. Proc. Roy. Soc. (London). 66:339-342.

Major, C.P., J.D. McDougal, and A.P. Harrison, Jr. 1955. The effect of the initial cell concentration upon survival of bacteria at -22 C. J. Bacteriol. 69:244-249.

Mazur, P. 1960 a. Physical and temporal factors involved in the death of yeast at sub-zero temperatures. Biophys. J. 1:247-264.

Mazur, P. 1960 b. Physical factors implicated in the death of microorganisms at subzero temperatures. Ann. N.Y. Acad. Sci. 85:610-629.

Mazur. P., M.A. Rhian, and B.G. Mahlandt. 1957 a. Survival of Pasteurella tularensis in sugar solutions after cooling and warming at sub-zero temperatures. J. Bacteriol. 73:394-397.

Mazur, P., M.A. Rhian, and B.G. Mahlandt. 1957 b. Survival of Pasteurella tularensis in gelatin-saline after cooling and warming at zero temperatures. Arch. Biochim. Biophys. 71:31-51.

McCleskey, C.C., and W.N. Christopher. 1941. Some Factors influencing the survival of pathogenic bacteria in cold-pack strawberries. Food Res. 6:327-333.

McFarlane, V.H., and H.E. Goresline. 1943. Microbial destruction in buffered water and in buffered sugar stored at -17.8 C. Food Res. 8:67-77.

Mehler, R.G., and S.E. Hartsell. 1963. A spectrum of a stimulatory factor from defrosted bacteria. Bacteriol. Proc., p. 7.

Meryman, H.T. 1960. General principles of freezing and freezing injury in cellular materials. Ann. N.Y. Acad. Sci. 85:503-509.

Meynell, G.G. 1958. The effect of sudden chilling on Escherichia coli. J. Gen. Microbiol. 19:380-389.

Moss, C.W., and M.L. Speck. 1963. Injury and death of Streptococcus lactis due to freezing and frozen storage. J. Appl. Microbiol. 10:40-43.

Nakamura, M., and D.A. Dawson. 1962. Role of suspending and recovery media in the survival of frozen Shigella sonnei. J. Appl. Microbiol. 10:40-43.

Nei, T. 1960. Effects of freezing and freeze-drying on microorganisms, p. 78-86. In Recent research in freezing and drying. Ed. by A.S. Parkes and A.U. Smith. Charles C. Thomas, Springfield, Ill.

Nelson, F.E. 1943. Factors which influence the growth of heat-treated bacteria. I. A comparison of four agar media. J. Bacteriol. 45:395-403.

Postgate, J.R., and J.R. Hunter. 1961. On the survival of frozen bacteria. J. Gen. Microbiol. 26:367-378.

Proom, H., and L.M. Hemmons. 1949. The drying and preservation of bacterial cultures. J. Gen. Microbiol. 3:7-18.

Prudden, T.M. 1887. On bacteria in ice, and their relations to disease, with special reference to the ice supply of New York City. Med. Record. 31:341-350,

369-378. (Cited from Weiser, R.S. and C.M. Osterud. 1945. Studies on the death of bacteria at low temperatures. J. Bacteriol. 50:413-439.

Reeves, N.C., and Harrison, A.P., Jr. 1957. Effect of time and temperature upon survival of Escherichia coli in sodium chloride. Proc. Soc. Exptl. Biol. Med. 95:278-282.

Rivers T.M. 1927. Effect of repeated freezing (-185 C) and thawing on colon bacilli, Virus III, vaccine virus, Herpes virus, bacteriophage, complement, and trypsin. J. Exptl. Med. 45:11-21.

Sanderson, E.S. 1925. Effect of freezing and thawing on the bacteriophage. Science. 62:377.

Smith, A.U. 1954. Effects of low temperatures on living cells and tissues, p. 1-62. In Biological applications of freezing and drying. Ed. by R.J. C. Harris. Academic Press, Inc., N.Y.

Smith,E.F., and D.B. Swingle. 1905. The effect of freezing on bacteria. Science. 21:481-483.

Squires, R.W., and S.E. Hartsell. 1955. Survival and growth initiation of defrosted Escherichia coli as effected by frozen storage menstra. J. Appl. Microbiol. 3:40-45.

Straka, R.P., and J.L. Stokes. 1959. Metabolic injury to bacteria at low temperatures. J. Bacteriol. 78:181-185.

Tanguary, A. E. 1959. Preservation of microbiological assay organisms by direct freezing. J. Appl. Microbiol. 7:84-89.

Tanner , P.W., and G.I. Wallace. 1931. Effects of freezing on microorganisms in various menstra. Proc Soc. Exptl. Biol. Med. 29:32-34.

Toyokawa, K., and D.H. Hollander. 1956. Variation in sensitivity of Escherichia coli to freezing damage during the growth cycle. Proc. Soc. Exptl. Biol. Med. 92:499-500.

Turner, T.B., and N.L. Brayton. 1939. Factors influencing the survival of spirochetes in frozen state.

J. Exptl. Med. 70:639-650.

Umbreit, W.W., R.H. Burris, and J.F. Stauffer. 1957. Manometric techniques, 3rd ed. Burgess Publishing Co., Minneapolis, Minn.

Weiser, R.S., and C.O. Hagriss. 1946. Studies on the death of bacteria at low temperatures. II. The comparative effects of crystallization, vitromelting, and devitrification on the mortality of Escherichia coli. J. Bacteriol. 52:71-87.

Woodburn, M.J., and D.H. Strong. 1960. Survival of Salmonella typhimurium, Staphylococcus aureus, and Streptococcus faecalis frozen in simplified food substrates. Appl. Microbial. 8:109-113.