THE CYTOCHEMICAL EFFECT OF FREEZING ON ESCHERICHIA COLI (ATCC#11303)

Thesis for the Degree of Ph. D.

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Edward F. Gritsavage

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THE CYTOCHEMICAL EFFECT OF FREEZING ON ESCHERICHIA COLI (ATCC#11303)

By

Edward F. Gritsavage

AN ABSTRACT OF A THESIS

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ABSTRACT

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ON ESCHERICHIA COLI (ATCC#11303)

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When suspensions of microorganisms are frozen and thawed, different percentages of survivors are recovered. Survival is affected by the nature of the suspending fluid and to the thawing procedure. Associated with this loss in number of cells is the release of intracellular constituents.

Microorganisms were suspended in various menstrua, frozen at -87C, subjected to a fast- and slow-thaw, and the menstruum analyzed for leakage products.

Solutions of monovalent cations afforded poor recovery whereas solutions of divalent cations gave moderate recovery. The effect of these cations appears to be related with the stabilizing effect they have on ribosomes. Suspensions that were slow-thawed gave smaller percentages of recovery than those that were fast-thawed.

A relationship between the percent recovery and the concentration of leakage products was established. Analyses showed the presence of a protein-like material thought to be low molecular weight peptides, pentose, and a substance that absorbed ultraviolet light. The U-V absorbing substance had

maximum absorption at 257 mu. The product was heat-stable, dialyzable, and could be adsorbed by and recovered from activated charcoal. Enzymatic assay with bovine pancreatic ribonuclease indicated that the product was not RNA. Fractionation of this material provided recovery in a barium-solube fraction. Absorption maxima, base ratios at 250:260 and 280:260 mu, and the recovery in this fraction indicated the material to be the nucleoside adenine.

The rate of color development of the pentose in the orcinol test indicated a 5' linkage of the phosphate to the pentose. Concentrations of the base:sugar:phosphate gave a ratio of 1:1:1 indicating the compound to be adenosine-5'-phosphate.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

Whether or not a suspension of microorganisms has been exposed to a stress, losses in viable number occur and different numbers of survivors are obtained. The survival of microorganisms under such conditions has been studied extensively, and many factors have been incriminated and considered as contributing to this phenomenon.

When a suspension of microorganisms is introduced into a new environment it is exposed to a variety of physical and chemical phenomena not present beforehand. It is the nature and effect of these phenomena that are concerned with survival, and, when this suspension of microorganisms is subjected to a stress, the problem of survival becomes more complex.

Under conditions of stress, biological injury to the cell results. This injury might be considered to be due to alterations produced within the cellular structure and metabolites. Consequently, it might be assumed that some of these alterations could be reversed, and if suitable means were applied, the cells might survive-- provided that the injury is in a range of tolerance. To increase survival, the nonreversal injury should be reduced or eliminated.

Recovery might apply in principle to all chemical and physical injuries produced within the cell, and restoration of viability might constitute a repair process, regrowth of structural elements, elimination of toxic materials from within the cell and its environment, or the formation of new adaptive structures. Many of the mechanisms involved in killing and protecting, as well as the responses under various conditions, are strikingly similar, intimating that the process of survival is related.

Probably the most extensively studied phase of survival is that of survival after exposure to freezing. In general, survival in the frozen state implies survival without growth. Past reviews on the effect of cold on microorganisms list the following observations: (1) On freezing, a sudden mortality varying with species exists, (2) the number of survivors after freezing is independent of the rate of freezing, (3) there is a gradual decrease in viability during frozen storage, (4) the decrease in number during storage is greater at temperatures near the freezing point, but less at lower temperatures and is lowest below -20 C, (5) death, or loss in viability, is usually greatest during the freezing process but is less during storage. Despite the volume of literature on this subject, it is difficult to find agreement

among the investigators as to the responsible mechanism.

Associated with freezing is a leakage of intracellular constituents. The purpose of this study is to characterize the nature of the leakage products and to determine its correlation with survival.

LITERATURE REVIEW

Effects of Suspending Media

Microorganisms stored in aqueous suspensions and subjected to no overt stress demonstrated that survival was influenced by conditions. Shearer (1917) reported that physiological saline solution was more toxic to meningococci than was distilled water or 1.5% NaCl solution. With studies on the mortality of 'Bacterium coli' and 'Bacterium typhosum' Cohen (1922) found that at constant temperature in unbuffered media like distilled water or tap water, the mortality was variable and coincident with apparently insignificant pH variations, but at constant pH, the resistance decreased with a rise in temperature. The tests of Sherman (1923) demonstrated that cells from old cultures were more resistant to brief exposures to cold, to mild heating, and the action of phenol. As a result of studies on salt action, Winslow and Falk (1923a, 1923b) reported that low concentrations of NaCl and $CaCl_2$ solutions had no effect on *E. coli* after 24 hours, but higher concentrations (0.725M NaCl; 0.435M $CaCl_2$) were toxic. When both salts were present the effects were additive. The toxic effect of $CaCl_2$ was attributed to an inability of the microorganism to reduce the alkalinity of the solution in which it was suspended.

Later, Winslow and Dolloff (1928) reported that an antagonistic effect of cations upon bacterial viability appeared only in a medium held at pH 7.0 and that the effects were additive in acid conditions. Further work of Winslow and Haywood (1931) on the effects of cations on viability showed that cations had a "specific potency" and had a general influence on the bacterial cell. This influence took the form of stimulation of viability (associated with increased permeability) when it was present in low concentrations and of inhibition (associated with decreased permeability) in higher concentrations.

Sherman and Cameron (1934) demonstrated that young cells of 'Bacterium coli' could be killed by abrupt environmental changes within the natural range of growth of the organism. In contrast, Hershey (1939a) found that the physiological state of the cells did not influence survival. Harrison (1961) observed that it did. The role of pH upon survival was considered when Gale (1943) observed that the enzymatic activities of a cell could be altered in response to alterations in the pH of the external environment. The production of amino acid decarboxylases was stimulated in acid pH while the production of deaminases was suppressed. Pinsky and Stokes (1952) found that adaptation of enzymes was favored in aging rather

than in physiological youth. Cook and Wills (1958) showed that washed E. coli suspended in phosphate buffer (0.1M; pH 7.0) maintained its viability better than did unwashed or washed cells suspended in water. Holden (1958) reported that incubation of Lactobacillus arabinosus in phosphate buffer at 37 C resulted in the degradation of RNA and the subsequent release of ultraviolet (UV) absorbing substances into the medium. Similar results were obtained by DeLamater, Babcock, and Mazzanti (1959) with Bacillus megaterium leached in 0.06M phosphate buffer, pH 7.4, for 2 hours at room temperature. Higuchi and Uemura (1959) obtained the release of nucleotides from yeast cells. Harrison (1961) held cells of different physiological states in phosphate buffer at 40 C and followed the loss of viability. Cells from slow log phase cultures survived better than cells from normal log phase cultures, and cells from stationary phase cultures survived best of all. Strange, Dark, and Ness (1961) studied the survival of populations of stationary growth phase Aerobacter aerogenes in non-nutrient buffer. They showed that the composition of the growth medium, the phase of growth, and the period of the stationary phase influenced survival. Death was preceded and accompanied by degradation and

excretion of polymeric cell contents (protein, ribonucleic acid, polysaccharide). Strange (1961) reported that these reactions degraded the ability of the organism to form adaptive enzymes. The observations of Postgate and Hunter (1962) showed that continuous cultures of A. aerogenes grown on a limited supply of glycerol died linearly with time without cryptic growth when aerated in buffered physiological saline solution at the optimum pH and temperature. Death was accelerated in environments of higher or lower tonicity, in unbuffered media, at pH values above 7, and temperatures above 40 C. Death was not attributed to a breakdown of the osmotic barrier. Survival was reported greater in dense populations than in sparser ones. dying populations also showed a rapid breakdown of intracellular RNA and the release of phosphate and base fragments into the medium; intracellular protein was reported degraded but intracellular polysaccharide and DNA scarcely degraded at all. A functional alteration of the cytoplasmic membrane was assumed to be the cause for the leakage of nucleotides from various microorganisms (Okabayashi, 1962). Later, Okabayashi, Yoshimoto, and Ide (1963) noted the excretion of a considerable amount of adenine ribonucleotide when Brevibacterium was grown

in a medium containing amino acids as the nitrogen source.

Several factors influence studies on the survival of microorganisms suspended in non-nutrient soultions. One factor is the presence of impurities in buffers that allow limited growth of bacteria (Garvie, 1955a,b). Another factor is the release of intracellular contents into the medium by dying populations supplying nutrients to the survivors. Ryan (1955) termed this 'cryptic growth.' Strange et. al. (1961) called it 'regrowth' and demonstrated that it could be prevented by dialyzing the suspension or renewing the suspending fluid after filtration.

Effects of Heat

Microorganisms exposed to heat exhibit varied responses. Curran and Evans (1937) found that the addition of blood or glucose to nutrient agar or the use of infusion agar usually increased counts of bacteria exposed to sub-lethal dosage of heat, ultraviolet light, and mercuric chloride. Hershey (1939b) also observed a related response when he noted that cultures of E. coli that were heated to temperatures near the thermal death point for 15 minutes gave lower survivor counts on nutrient agar than by serial dilution in nutrient broth. A continuous

decline in resistance level in younger cells of E. coli that were exposed to heat was noticed by Ellicker and Frazier (1938) while cells in the logarithmic phase showed lower resistance. Lemcke and White (1959) demonstrated that cells harvested from broth cultures 0-8 hours old were more susceptible to heating at 55 C than those from more mature cultures. The observation that bacteria subjected to sub-lethal heat were more demanding in their growth requirements than were unheated controls (Nelson, 1943) lead to possible explanations for these varied responses. The first was that of Califano (1952) who noted that exposure to heat caused a separation of ribonucleic acid in soluble form from the bacteria that diffused into the suspending fluid. Strange and Shon (1964) studied the effects of thermal stress on viability and presented data that take into account many factors. They found a complex mixture of substances, one of which absorbed ultraviolet light, present in the suspending fluid after exposure to heat. The addition of these leakage products to the heated suspension did not increase the viability or recovery of the bacteria. In the treatment of their cells, Strange and Shon (1964) found that washing in salt solutions desorbed magnesium resulting in a higher death rate of

bacteria compared with bacteria washed in distilled water. The addition of magnesium to the diluent in which the bacteria were heated largely eliminated the differences in thermal resistance resulting from the pre-washing treatment. A relationship between the rates of death and of RNA degradation is intimated since magnesium, which stabilized isolated ribosomes for Mc Quillen (1962), decreased the rate of death.

Effects of Ultraviolet Irradiation

The increase of the survival capacity of the cell by reversal of alterations produced within the cell can be demonstrated when cells are inactivated with ultraviolet irradiation. Kelner (1949) was one of the first to show that strains of E. coli, inactivated by exposure to ultraviolet irradiation, could be reactivated by exposure to normal light. About the same magnitude of reactivation was reported by Anderson (1951) who reactivated irradiated E. coli with heat. Thompson, Mefferd, and Wyss (1951) failed to recover E. coli from U-V injury by adding pyruvate to the cells. Their intention was to oxidize the hydrogen peroxide formed during irradiation. However, the enzyme catalase, which oxidizes hydrogen peroxide, induced restoration

to cells of E. coli, strain K-12, inactivated by U-V irradiation (Latarjet and Caldas, 1952). Heinmets (1953) was successful in reactivating irradiated cells incubated in pyruvate solution. In later experiments Heinmets and his co-workers (1954a, 1954b) found that the most effective reactivation agents for irradiated cells were various metabolites belonging to the tricarboxylic acid cycle. Amino acids, with the exception of alanine, were reported to have only a moderate effect. Studies on the effects of irradiation of microorganisms in relation to food preservation (Erdman, Thatcher, and Mac Queen, 1961) showed that sensitivity was influenced by the nature of the suspending medium during irradiation. In a different area of study, Stapleton and Engle (1960) reported that radioresistance (resistance to inactivation by x-rays) of E. coli strain B/R, parallels relative resistance to the U-V irradiation and thermal stress.

Effects of Chemical Agents

Permeability defects have been reported as causes for the decrease in survival of organisms treated with detergents, chemicals, and drying. Slade (1957) reported losses as great as 99% when cells of Streptococcus pyogenes were treated with a cationic detergent cetyltrimethylammonium bromide.

These losses were accompanied with the diffusion of amino acids, primarily lysine and glutamic acid, from the cells. Strauss (1961) produced a similar effect in cells of E. coli treated with ethyl sulfate. Once more, there was a decrease in number which was followed by an excretion of ultraviolet absorbing material into the medium and a decrease in intracellular nucleic acid. The killing and excretion could be prevented by the addition of magnesium to the incubation medium. It is interesting to note the stabilizing effect magnesium has on protoplasts (Weibull, 1956) and on spheroplasts (Lederberg, 1956; McQuillen, 1958) suggesting that these ions might protect bacteria during freezing.

In studies with disinfectants, Wright (1917) concluded that the medium in which the organism was grown before being acted upon by disinfectants was much more important than the recovery medium in determining ability of organisms to survive. Jacobs and Harris (1960) maintained the opposite. Cells damaged by phenols continued to decrease in number after being transferred to nutrient broth but there was no such decrease if the broth contained Norit, an activated charcoal. Later Jacob and Harris (1961) reported that media, both solid and liquid, when treated with Norit before inoculation with cells

damaged by phenol, gave better results than media that wasn't treated. These findings supported their view that the medium contained materials that were toxic to the bacteria.

Effects of Desiccation

In the preservation of bacteria by drying, Stamp (1947) found that survival rates depended upon the medium used and the method of drying. Record and Taylor (1960) showed that organisms that were dried in sucrose solutions were subject to internal diffusion pressures on reconstitution. Such diffusion pressures could cause disruption and death of the organisms. Increased permeability of the cell wall of yeast upon drying was reported by Ebbutt (1961) who considered this as of greater significance as a cause of loss of activity.

The most commonly used method of preserving microorganisms is lyophilization (freeze-drying) but this is a drastic procedure for in most instances only 2-3% of the organisms survive the process. Cultures preserved in this way have remained viable for as long as 35 years (Engley, 1956). The loss in number is attributed to altered permeability of the cell membrane and destruction of enzymes (Wasserman and Hopkins, 1958).

In studies on the survival of E. coli, Record and Taylor (1953) found that a relationship existed between the percentage of survivors and the initial concentration of organisms in the suspension; the more dilute the suspension, the lower the percentage of survivors. The dependence of survival was shown to be due to soluble material derived from the organisms but protection by the material was afforded in the drying state only. Clement (1961) obtained high survival in frozen and partly dried specimens but viability decreased with continued drying and further losses occurred when the preparations were dry. Wagman (1960) presented evidence of cytoplasmic injury as the primary effect of drying in bacteria. Suspensions of E. coli were freeze-dried in water and allowed to stand for 3 hours. A subsequent decrease in numbers was obtained as well as the release of ultraviolet absorbing material and amino acids.

Effects of Freezing

The general topic of the loss in viable numbers of microorganisms frozen in suspensions has been reviewed extensively. Despite an extensive study, differences of opinion exist among the investigators as to the cause. As a result, a multitude

of theories has been presented.

In the early work of Macfadyen and Rowland (1900) a variety of organisms were frozen at -190 C for 7 days and growth was obtained with every type. However, no quantitative data were reported. Perhaps the earliest quantitative report was that of Smith and Swingle (1905) who obtained up to 99.98% kill of 'Bacterium typhosum' in broth after freezing at -17.8 C. No increase in bacteria in milk held at -9 C was noted by Ravenel, Hastings, and Hammar (1910) whereas there was a marked increase in milk held at 0 C. Escherichia coli suspended in a variety of liquids and frozen at -20 C yielded different percentages of recovery (Keith, 1913). Tap water was reported to be more lethal while milk and glycerine provided protection. The death in water was considered due to mechanical crushing. Hilliard, Gorossian, and Stone (1915) suspended organisms in milk and subjected them to fluctuations in freezing temperature and found that -15 C was more fatal than -2 C. Later Hilliard and Davis (1918) suspended bacteria in various menstrua. Some samples were frozen and refrozen and some were suspended in a medium that did not freeze but reached sub-zero temperatures. From their data they concluded

that (1) intermittent freezing exerts a more effective germicidal action than continuous freezing; (2) the reduction is less in milk and cream than in pure tap water when freezing temperatures are applied; (3) the degree of cold below freezing is not a very important factor in the destruction of bacteria. There is a critical temperature below freezing where the germicidal effect is greatly accelerated; (4) the death rate of E. coli is higher in media which is frozen solid than it is in the same media not solid and at a slightly lower temperature; (5) crystallization, probably resulting in mechanical crushing, is an important germicidal factor in causing the death of bacteria at 0 C. The greatest reduction occurs promptly upon freezing and refreezing. Paradoxically, Sanderson (1929) subjected bacteriophage to repeated freezing and thawing and obtained no loss in titer, and Smart (1935) observed slight growth in cultures of microorganisms stored at about -9 C over a year, indicating that some biological activity is possible. Berry (1933) presented data showing a decrease in survivors of suspensions held at various temperatures. At -20 C, a 40% decrease was found; 99% at -10 C, and over 99% at -2 C. Using a variety of

organisms and freezing them on agar slants, beef tea, and on sterile filter paper at temperatures of liquid air for one week, Winchester (1936) found that all remained viable. Turner and Brayton (1939) noted that storage temperatures of -20 C, -10 C or warmer decreased virulence for spirochetes but at a storage temperature of -78 C there as no demonstrable loss of virulence. These investigators maintained that injury arises from two sets of factors: the act of freezing and thawing, and the other with storage. A more extensive study of this by Meiser and Osterud (1945) lead them to believe that death by freezing involves an "immediate" death caused by freezing and thawing, and a storage death which is a direct function of time and temperature. The mortality due to immediate death by freezing was marked but did not vary with the intensity of the freezing temperature. This immediate death occurs at a brief stage during which external ice is being completed. The rate of storage death is higher at temperatures above -30 C.

Only two factors were considered by Proom and Hemmons (1949) to influence the survival rate after freezing: (1) the species, and (2) the nature of the suspending medium. They believed that extra-

cellular ice crystals punctured the cell wall and that different species exhibited differences in the strength of the cell wall. Saline solution was found to produce the greatest reduction in numbers while gelatin decreased the loss. The protection afforded by gelatin was attributed to the formation of colloids around the cells that protected the organisms against damage from ice crystals.

The protective power provided by glycerol was demonstrated by many investigators. Lovelock (1953) obtained protection when red blood cells were frozen in the presence of glycerol. However, he maintained that glycerol was protective only when it had permeated the cell before freezing and it did not protect against osmotic or thermal shock. Cells impermeable to glycerol are not likely to survive even in its presence. Hollander and Nell (1954) theorized that the protection offered by glycerol against the effects of freezing could be explained by its physical characteristics. They believed that death resulted from mechanical compression. Additional reports on the protection by glycerol come from Howard (1956) who maintained viability of cells frozen in glycerol and stored at -10 C for up to 5 months, and from Quadling (1960) who found the glycerol provided protection for

Xanthomonas and no differences were found when thawing was varied. Tanguay (1959) preserved microbiological assay organisms by freezing in glycerol, and Floodgate and Hayes (1961) used it to preserve marine bacteria.

Increased survival in other menstrua has also been reported by various investigators. Squires and Hartsell (1955) indicated that survival was related to storage menstruum and that stimulatory materials accumulated during frozen storage. Suspensions of organisms in lactose-phosphate buffer solution had remained viable for Mead et. al. (1960) after one year of storage at -23 C.

Several factors have been suggested as influencing survival. Major, McDougal, and Harrison (1955) stored eleven species of bacteria in broth at various cell concentrations at -22 C and found that in most cases, survival varied in proportion to the initial cell concentration. Later, Harrison (1955) reported that with Lactobacillus, the percentage of survival after a series of repeated freezings and thawings was dependent upon the initial cell concentration. Lion (1961) suggested that loss of viability occurred during the period when oxygen was in contact with cells.

The growth phase of the culture has also been mentioned in connection with survival. Morse and Carter (1949) observed a large increment in RNA during log phase. Cells in this growth phase were more susceptible to heat, cold, and U-V light. While information on the connection between these activities and nucleic acid is limited, a connection is intimated. Toyokawa and Hollander (1956) noted that E. coli varies in its sensitivity to damage and reduction in freezing and that cultures in the log phase of growth were more susceptible than older cultures. Organisms in the stationary phase of growth were reported to be completely resistant to freezing in 0.3M sucrose solution (Meynell, 1958) and that susceptibility of growing organisms changed rapidly during the exponential growth phase. The response was not due to the chilling in itself but required a suitable diluent as well. The subsequent evidence of Gorrill and McNeil (1960) substantiated this view and they also stated that in general the simpler the composition of the diluent, the more lethal it was.

During the period of years in which studies on freezing were conducted, a number of theories appeared which attempted to explain the mechanisms

involved in protection and injury of microorganisms during freezing.

One of these was the theory of death by crushing which was proposed by Keith (1913) and later by Haines (1938) who found no evidence to support this theory. Using direct measurements, no changes in size were discerned in bacteria that were frozen. Haines suggested that coagulation of protein was responsible for the death of bacterial cells on freezing. Further evidence of Harrison and Cerroni (1936) discounted the credence of such a theory because no relationship between the cells and susceptibility to freezing was found. The lethal factor is considered by them to be something other than mechanical.

Dubos (1937) attributed the death of frozen Pneumococci to the activity of autocatalytic enzymes.

In studies on the death of bacteria at low temperatures, Meiser and Hargiss (1946) proposed that during ice formation, the concentration of the solute surrounding the cell was higher than within the bacterial cell and dehydration took place. Similar results were indicated by Harrison (1956) who demonstrated similarities between the behavior of bacterial cells in frozen and unfrozen solute.

The conclusion was that death in frozen suspension has to do with concentration of the solutes upon solidification.

Such a theory would gain support from the work of Luyet (1960) who studied phase transitions occurring in aqueous solutions at low temperatures. He showed that in dilute solutions, solidification took place in two steps: a first in which the solvent freezes out while the solute becomes more concentrated; and a second, in which the components of the concentrated solution solidify.

However, the results of Mazur, Rhian, and Mahlandt (1957a, 1957b) favor a theory of death due to the formation of intracellular ice. When cells of Pasteurella tularensis were cooled to temperatures between -15 C and -75 C, recoveries after slow warming depended upon the rate of cooling. These findings, they concluded, were compatible with the hypothesis that recovery was related to the extent of intracellular freezing. Wood and Rosenberg (1957) reported survival in yeast even though as much as 90% of cellular water was frozen. Intracellular ice was considered to be a pre-requisite to death during slow warming and those factors which lessened the injurious effects of slow warming did so by reducing the extent of intra-

cellular freezing. Later, Lazur (1960) presented information strengthening this theory and helped to discount the theory of increased concentration of solute as a cause. His data showed that the drop in survival was less extensive when the cooling was slow than when cooling was rapid. He concluded that death was not the result of high concentrations of solute. If it were, longer exposures during slow freezing would have produced greater damage and this did not happen.

Because lipid-protein complexes make up cell membranes, Lovelock (1957) proposed an additional theory. Since these complexes are held together by weak association forces instead of strong co-valent bonds, he attributed rupturing of these bonds as the result of freezing. This was accomplished in freezing by (1) increased electrolyte concentration, (2) changes in pH, particularly if sparingly soluble buffering salts crystallize, and (3) removal of water. This theory was based on the observation that membranes of red blood cells lost phospholipid when suspended in various molar concentrations of NaCl solutions. A loss of solubility was used as the criterion of denaturation and human plasma lipoprotein was denatured when frozen.

Mager et. al. (1956) detected changes in the turbidity of bacterial suspensions with variations of the osmotic pressure of the suspending medium. These changes were dependent upon the viability of the cells. Since heating and freezing-thawing reduced both viability and the optical effect, the reduction was attributed to osmotic pressure changes consequent to freezing. Bretz and Hartsell (1959) observed that some bacteria showed osmotic sensitivity ('osmosensitivity') after freezing and thawing since populations gave greater recoveries of viable organisms when diluted in 20% sucrose solution before plating than when diluted in ordinary buffer solutions. Since materials such as the diamines of Mager (1959), which protect osmotically fragile organisms, are likely to be present in a rich medium as opposed to a minimal medium, osmosensitivity might provide an explanation for metabolic injury. Unfortunately, Postgate and Hunter (1961, 1963a,b) could not demonstrate this optical effect in suspensions of Aerobacter and their findings were incompatible with the view that the lethal effects of freezing were connected with osmotic shock.

Probably the most tenable theory was that suggested by Hartsell (1959). From the assumption that some synthesis is possible even at sub-freezing temperatures (Smart, 1935), products should accumulate as the cells are held in storage and later isolated. This presented the foundation for the theory that freezing causes "metabolic injury" to microorganisms. Straka and Stokes (1959) supported this theory with the observation that cold injury was manifested by an increase in nutritional requirements, and that the active substance required by injured cells was peptides. This was also demonstrated by Bretz and Basa (1960) with greater recoveries of cells on complete media than on minimal media. The mechanism that might explain this requirement was indirectly suggested by Mandelstam (1960). In bacteria, cells that have expended one substrate in the growth medium are engaged in producing an inducible enzyme for the utilization of another. This "metabolic injury" can be explained as a result of protein turnover. This theory was strengthened by the results Bretz (1961) reported. He discounted the theories of "mechanical crushing" and "concentrated solute" as well, when he observed that E. coli frozen on membrane discs or cellophane gave similar responses

as cells in frozen suspension. There was an increased survival of cells regardless of the method of storage which implied an interaction between cells prior to freezing.

Arpai (1962) indicated that non-lethal metabolic freezing was related with time, temperature, and the nature and pH of the suspending fluid. The role of the suspending and recovery medium upon survival after freezing was shown by Nakamura and Dawson (1962) and supported the view that freezing alters the nutritional requirements of microorganisms.

Associated with protein turnover is a subsequent turnover of RNA. Mandelstam (1960) reported that the instability of RNA seemed to be a consequence of stopping growth. Under conditions of starvation, the ribosomes disintegrate either because there is a decrease in the concentration of Mg^{++} or for some other reason. A solubilization of the ribonucleoprotein occurred and the RNA which was protected in the particulate state became exposed to attack. The breakdown of RNA was indicated by the appearance of an acid-soluble material absorbing at 260 mu.

Chilled suspensions of Aerobacter were found by Strange and Dark (1962) to "leak" free amino

acids, low molecular weight peptides, a substance that absorbed U-V light, but no acid soluble RNA could be detected. Similar results were observed by Warfringe (1962) who subjected a suspension of living tubercle bacilli to repeated freezing and thawing and extracted a material that could sensitize guinea pigs to tuberculin. Biochemical and electrophoretic studies showed several proteins, two polysaccharides, lipid, and a high concentration of nucleic material. Ambrosini and Dretz (1963) prepared extracts from washed E. coli by freezing and thawing, boiling, or by autoclaving that yielded a protective factor for the increased survival of E. coli that were frozen and thawed. Survival correlated with the carbohydrate content; fractionation gave anomalous results and Norit treatment or dialysis increased its activity. Roszman (1963) presented evidence that extracts obtained from frozen and thawed E. coli stimulated oxygen uptake and observed a decrease in activity when the extract was dialyzed or treated with Norit. Mehler and Hartsell (1963) isolated a growth-stimulating substance, "Factor S", from frozen E. coli which appeared to be non-specific in its activity. A loss in viability was accompanied by a release of U-V

absorbing material when Lindeberg and Lode (1963) cooled suspensions of E. coli in artificial seawater to subzero temperatures. Postgate and Hunter (1963) showed more viable bacteria partly killed by freezing and thawing when plated on a rich medium than on a poor one. The presence of toxic materials in agar as suggested by Jacobs and Harris (1961) was not contributing to the decrease in numbers. The addition of $FeCl_3$ and Norit to the rich recovery medium to remove toxic materials gave results insignificantly different from those on minimal media without Norit.

MATERIALS AND METHODS

Bacterial strains

Escherichia coli (ATCC #11303) obtained from the Division of Laboratories, Michigan Department of Health and *Aerobacter aerogenes* obtained from Miss Lisa Neu, Department of Microbiology and Public Health, Michigan State University, were used throughout this study.

Culture maintenance

All cultures of the test organisms were maintained by semi-monthly transfer on tryptone glucose extract agar (Difco) slants and stored at 4 C. Cells for tests were grown on the same medium, either on slants or in vaccine jars when large quantities of cells were required. Cultures were incubated at 37 C for 22-24 hours. Vaccine jars were inoculated by spreading 10 ml of a suspension of cells harvested from agar slants over the surface of the medium. All cells were taken from stock cultures and transferred at least once before being used as an inoculum.

Preparation of cell suspensions

Cells were harvested from the agar by "flooding" with sterile physiological saline solution (0.85% NaCl). Cells were pooled into sterile centrifuge

tubes and centrifuged at room temperature at 22,000 x g for 10-15 minutes. The liquid was decanted and the cells resuspended in 10ml of the sterile physiological saline solution. This procedure was repeated three times. After the final centrifuging, the pellet was washed with a small amount of the diluent and re-suspended in 10ml of the diluent.

Assay of cells

Assays of viable organisms in suspension before and after testing were determined by making 10-fold serial dilutions in 0.85% saline solution. Triplicate pour plates of each dilution were made and incubated at 37 C for 48 hours before counting. Viable organisms will be used to designate those organisms that produced visible colonies on the particular medium used. Percentage survivors were determined by using the following equation:

$$\% \text{ Survivors} = \frac{\text{\# organisms after treatment}}{\text{\# organisms before treatment}} \times 100$$

Preparation of extracts

Extracts were prepared by freezing a suspension of organisms, storing at sub-zero temperature, and thawing. Following thawing, the suspension was thoroughly mixed by aspiration with a pipette,

a volume of 1.0ml taken for assay, and the remainder centrifuged at 22,00 x g at room temperature for 25-30 minutes. Extracts were freed from bacteria by filtration through sterile membrane filters and stored in sterile, screw-cap tubes at 4 C except when used for analysis.

Analysis of extracts

Extracts were analyzed for pentose concentration by the Bial reaction (Albaum and Umbreit, 1947) with D-ribose as standard; DNA by Burton's (1956) modification of the diphenylamine reaction with thymus DNA as standard. Protein concentration in the extracts was determined by the Lowry (1951) modification of the Folin reaction with bovine serum albumin fraction IV as standard. Inorganic and seven-minute hydrolyzable phosphate were determined by the Fiske-Subbarow (1925) method with KH_2PO_4 (1u mole/ml) as standard. Amino acids were detected using the modified ninhydrin reagent of Moffat and Lytle (1959) following ascending paper chromatography. Ultraviolet absorption (U-V) spectra were obtained with a Beckman spectrophotometer, model DU, with a 1.0cm light path. Colorimetric determinations were performed in matched tubes for optical cells using a Bausch and Lomb "Spectronic 20"

photometer. Concentrations of reacting materials were determined by extrapolation from standard curves which were plotted using concentrations that would follow the Beer-Lambert Law. Quantitative spectrophotometric analyses where only a single absorbing compound was present were determined using the following equation:

$$c = \frac{D}{k'}$$

where k' is the specific extinction (absorption) coefficient, D is the optical density of the unknown at the wavelength k', and c is the concentration expressed in moles per liter, and the light path is 1.0 cm. The extinction coefficient for AMP used in this study is 14,900 $M^{-1}cm^{-1}$ at 257 mu.

Fractionation of extract

Fractionation of the extract was performed using the method for the analysis of phosphorylated intermediates outlined by Umbreit, Burris and Stauffer (1959). The following procedure was used: to 10ml of the cell extract obtained from concentrated suspensions of frozen cells, an equal volume of cold, 10% trichloroacetic acid (TCA) was added, mixed, and allowed to stand at 4 C for 30 minutes. The precipitate was removed by centrifuging at 22,000 x g

for 30 minutes at 4 C and the supernatant liquid decanted and saved for further fractionation. The precipitate constituted the TCA-insoluble fraction. This was washed with saline solution and dissolved in 1N NaOH for spectrophotometric and colorimetric analysis if necessary.

The supernatant liquid was extracted twice with 10 ml of ethyl ether in a separatory funnel and the aqueous phase collected. Excess ether was removed by heating in a steam bath until no odor of ether could be detected. This constituted the TCA-soluble fraction.

After the removal of TCA, a spectrum was determined and the remainder of the acid-soluble fraction was further separated by the use of the solubilities of the barium salts at pH 8.2. Concentrated solutions of reagents were used to prevent appreciable increase in volume of the fractions.

The acid-soluble fraction was adjusted to pH 8.2 with 3N KOH and excess barium added in the form of crystalline barium acetate until saturation was reached. The extract after the addition of barium (at pH 8.2) was chilled at 4 C for 15 minutes and the precipitate removed by centrifugation at 22,000 x g for 30 minutes at 4 C. The precipitated material constituted the barium-insoluble fraction

whereas the supernatant fluid constituted the barium soluble fraction.

The precipitate (barium insoluble) was dissolved in 1N HCl and the barium removed with a slight excess of 10N H_2SO_4. The precipitated barium sulfate was removed by centrifugation. The supernatant liquid was checked for the presence of barium by the addition of a drop of H_2SO_4 and an absorption spectrum of the barium free liquid was made.

The barium soluble fraction was treated with 10N H_2SO_4 to remove the barium as the sulfate which was removed by centrifugation and the supernatant liquid analyzed spectrophotometrically. Following spectrophotometric analysis the barium soluble fraction was checked for pentose and phosphate concentrations.

Blanks for both colorimetric and spectrophotometric determinations were obtained by treating a volume of the diluent used to prepare the extract to the same fractionating procedure.

Suspending fluids

The following solutions were used to suspend the organisms for freezing: (1) 0.5M phosphate buffer (12 ml M/5 Na_2HPO_4; 460 ml M/5 NaH_2PO_4; distilled water to 1000 ml) pH 5.3, (2) 15% glycerol, (3) 0.2 M magnesium acetate, (4) 0.02 M magnesium

acetate, (5) 0.002 M magnesium acetate, (6) distilled water, (7) 0.02M sodium acetate, (8) 0.2 M citrate buffer (2.101 g. citric acid monohydrate; 20 ml M/1 NaOH; make up to 50 ml and dilute with an equal volume of distilled water) pH 5.0, (9) 0.2 M sodium acetate, (10) 0.85% sodium chloride solution.

Freezing and thawing

Cell suspensions were frozen in either sterile 20x150 mm glass test tubes or in large, sterile, plastic centrifuge tubes. Glass test tubes were placed in a slanted position during freezing to avoid breakage. Freezing and storage of suspensions were done in a freezer chest set at a temperature of -87 C. Time lapse during this process was generally 8-12 hours. Thawing was accomplished either by immersion with gentle agitation in a 55 C water bath (fast-thaw) or by standing at room temperature or refrigerator (4 C) temperature (slow-thaw). Fast thawing required about 2-3 minutes and slow thawing from 15-30 minutes to several hours depending upon the volume of the suspension. Following thawing, the suspensions were mixed and viable cells determined by the previously described method.

Preparation for time and thawing experiment

A washed suspension of cells in saline solution

was serially diluted in 0.85% NaCl solution. As many 5.0 ml volumes as possible of each dilution were pipetted into sterile 20x150 mm test tubes. Tubes of each dilution were divided into two sets. One set was placed in a refrigerator at 4 C and the other set frozen at -87 C. After various time intervals two tubes of each dilution were removed from the freezer. One set was "fast-thawed" and the other set "slow-thawed" at room temperature. Triplicate samples from each dilution of the refrigerated and frozen-thawed suspensions were assayed for viable cells using the previously described technique.

An undiluted suspension of cells was divided into two equal volumes and frozen at -87 C. One tube was fast-thawed and the other slow-thawed at 4 C. Cells were mixed thoroughly, a suitable volume taken for assay, and the extract removed by the described method. Extracts from both treatments were diluted 1:5 with 0.85% saline solution and an absorption spectrum noted.

Paper chromatography

Whatman number 1 filter paper was employed in the chromatographic separation of the amino acids in the cell extract. Sheets 40 x 57 cm were used to allow simultaneous chromatography of 20 amino acids

used for identification. Samples 5 mm in diameter were applied to the paper with capillary tubes and allowed to air dry at room temperature. The paper sheet was rolled into a cylinder and placed in a chromatography jar lined with filter paper saturated with solvent. The solvent used was n-butanol-glacial acetic acid-water (4:1:5 v/v). After the solvent front had migrated a sufficient distance, the paper was removed, the solvent front marked, and the paper dried in an oven at about 120 C. Amino acids were detected by spraying with modified ninhydrin reagent (Moffat and Lytle, 1959). Chromatographic identification was obtained by comparing known amino acids to those in the extract.

Electrophoresis

Cellulose acetate strips (Oxoid Division, Oxo Ltd., London) were employed for electrophoresis. A 5 lambda volume of the cell extract was applied to the strips by means of a micropipette. Electrophoresis was carried out in a refrigerator (4 C) for 2½ hours at a potential gradient of 1 milliamp per strip. Owen buffer (5.0 g. sodium diethyl barbitone; 3.25 g. sodium acetate (hydrate); 34.2 ml 0.1 N HCl; distilled water to 1000 ml) pH 8.6 was used as the solution. Strips were removed, fixed

in 20% sulfosalicylic acid for 1 minute, stained in Coomassie Brilliant Blue R-250, (0.25% in distilled water), for 5 minutes and differentiated in distilled water.

Pervaporation

A 10 ml volume of extract was placed in cellulose dialysis tubing and pervaporated in the air stream of a fan at room temperature until the volume was reduced to 1/10 of the original volume. The volume was measured, brought to the original volume by the addition of 0.85% saline solution, and an absorption spectrum determined.

Heat stability

Two 1:5 dilutions of the extract were made in 0.85% saline solution. One dilution was heated in a boiling water bath at 100 C for 15-20 minutes. The precipitate was removed by centrifugation at 22,000 x g for 15 minutes. An absorption spectrum of the supernatant fluid and the unheated dilution was made.

Adsorption

One-tenth gram of Norit (Pfanstiehl Chemical Co., Waukegan, Ill.), autoclaved at 121 C, 15 lb./in^2 pressure for 15 minutes and activated by heating

to cherry-red in a bunsen flame, was added to 10 ml of the extract. One-tenth ml of 95% ethyl alcohol was added to reduce surface tension and the mixture placed on a magnetic stirred at 4 C for 18-24 hours. The Norit was removed by gravity filtration and the supernatant fluid saved for spectrophotometric analysis.

The Norit was washed on the filter paper three times with 0.85% NaCl solution. The adsorbed material was eluted from the charcoal with 25 ml of a solution containing absolute ethyl alcohol-concentrated ammonium hydroxide-distilled water (25:1:24 v/v). An absorption spectrum of the eluate was determined.

The eluate was evaporated to dryness at room temperature and the resultant crystals resuspended in both 1N HCl and 1N NaOH. Absorption spectra were plotted and the base ratios at 250:260 and 280:260 mu were determined.

Enzymatic assay

The presence of ribonucleic acid in the cell extract was determined by the method of Bingham and Zittle (1962). The assay mixture contained 0.65 ml veronal-acetate buffer (pH 7.5), 0.25 ml 1% yeast RNA or 0.25 ml of cell extract, and 0.1 ml of bovine pancreatic ribonuclease. Increase in

optical density at 260 mu was used as a measure of ribonuclease activity.

Sonic rupturing

Cell suspensions, prepared in the previously described manner, were placed in sterile plastic centrifuge tubes and exposed to sonic vibrations in a Raytheon Sonic Oscillator, Model 250, for 30 minutes. Cellular debris was removed by centrifugation and filtration and the supernatant fluid tested for the presence of DNA.

RESULTS

Occurrence of Cell Extracts

Effects of freezing and thawing

When suspensions of microorganisms are frozen and thawed, different numbers of survivors are obtained. The death of the microorganisms is accompanied by the degradation and release of cell contents into the suspending medium. These products consist of pentose, a product that absorbs ultraviolet light, and protein-like material. Analysis of the U-V material showed that maximum absorption at pH 7.0 was at 260 mu with base ratios at 250:260 and 280:260 of 0.90 and 0.50 respectively (Figure 1). No deoxypentose could be detected, and paper chromatography showed no free amino acids; ninhydrin-positive materials thought to be low molecular weight peptides were detected.

Suspensions of *E. coli* were ruptured by sonic vibration and the extracts tested for the presence of deoxypentose. Positive reactions were obtained for six trials. The absence of deoxypentose in the extract obtained from frozen cells would discount the possibility that death was due to rupturing of the cell membrane and is incompatible with a theory of death by crushing.

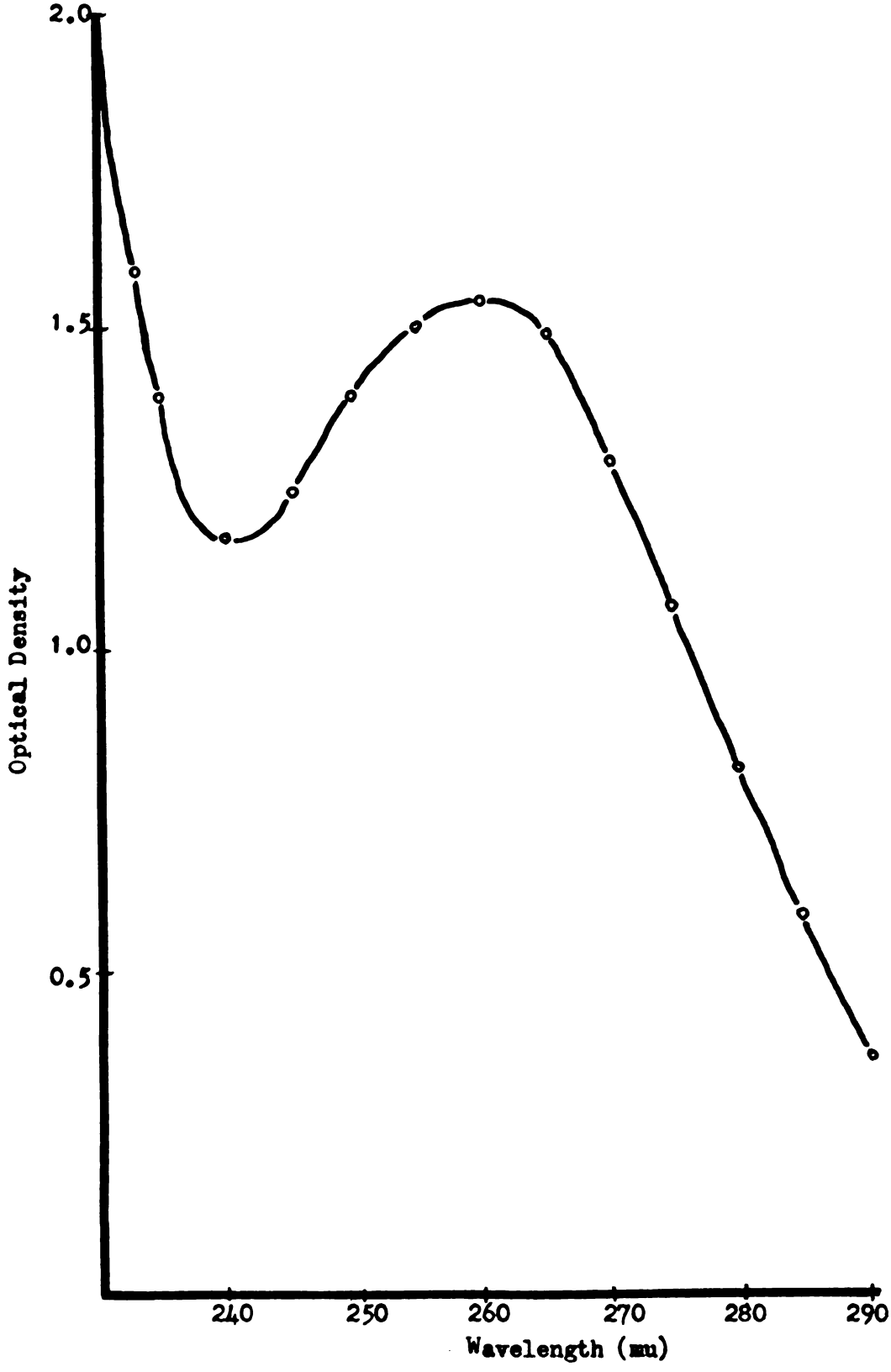

Fig. 1. Absorption spectrum of cell extract obtained from frozen suspensions of Escherichia coli (1:5 dilution)

Relationship between recovery rate and concentration of leakage products

Suspensions of E. coli were subjected to freezing and slow thawing in saline solution to determine the percentage of survivors and the concentration of leakage products. One series was analyzed for protein and pentose and another series for absorption at 260 mu. The results are given in Table 1.

These results indicate that frozen cells lose viability at a greater rate than chilled (4 C) suspensions and that the concentration of leakage products obtained from the frozen cells is greater than that from chilled suspensions. From this, it can be concluded that a relationship exists between the percentage of survivors and the concentration of the leakage products in the suspending medium.

The leakage of cell constituents which occurred as a result of freezing suggested that a permeability control mechanism was affected by freezing. Such a mechanism would presumably be located in the cytoplasmic membrane.

The effects of the nature and concentration of various menstrua on the survival of E. coli in frozen suspension are shown in Figure 2. These data indicate that monovalent ions are more lethal than divalent ions.

Table 1. The percentage of survivors and concentration of leakage products after freezing Escherichia coli in saline solution at -87 C for 4 hours and slow thawing at 4 C for 12 hours.

Trial Number	% survivors	Pentose (ugms/ml)	Protein (ugms/ml)	O.D at 260 mu (1:5 dilution)
1	1.26	52	6.7	-
2	2.23	34.5	5.8	-
3	1.55	49	6.7	-
4	1.60	36.5	4.5	-
5	8.0	13.5	1.5	-
6	3.38	-	-	0.675
7	3.02	-	-	1.00
8	1.83	-	-	1.25
*9	86.0	3.0	0.325	-
*10	78.0	3.75	0.45	-

* Indicates cells suspended in saline solution and stored at 4 C for 18 hours and subjected to no freezing and thawing.

(1) 0.5M phosphate buffer, pH 5.3
(2) 15% glycerol
(3) 0.2M magnesium acetate
(4) 0.02M magnesium acetate
(5) 0.002M magnesium acetate
(6) distilled water
(7) 0.02M sodium acetate
(8) 0.2M citrate buffer, pH 5.0
(9) 0.2M sodium acetate
(10) 0.85% sodium chloride solution

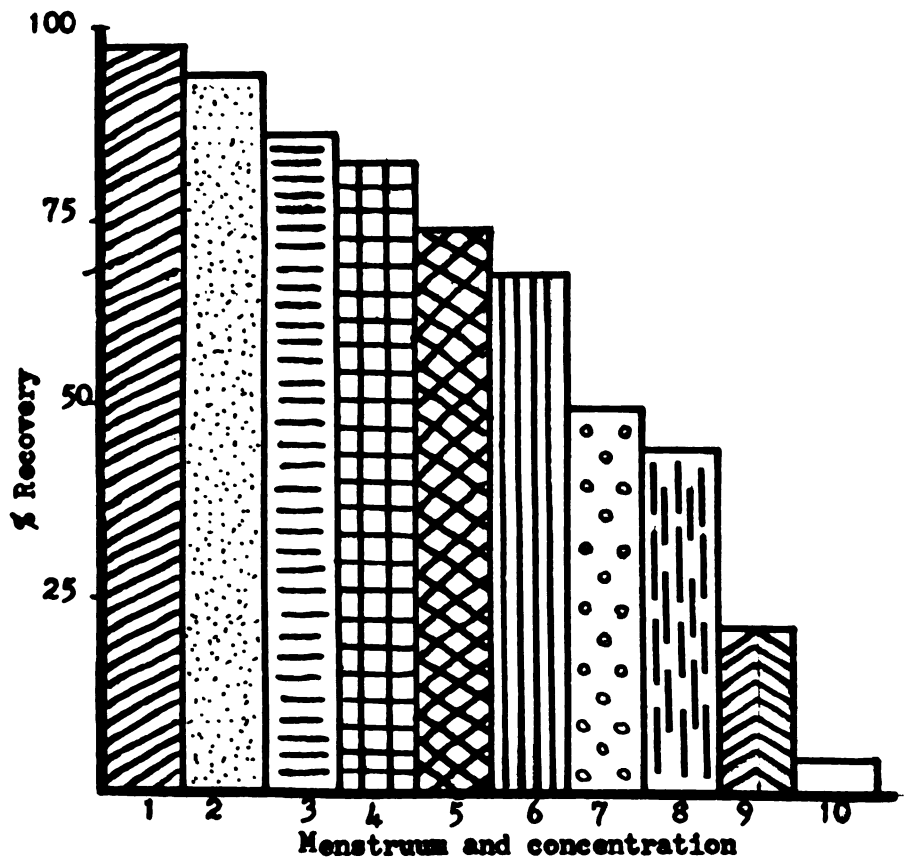

Fig. 2. Recovery of Escherichia coli subjected to freezing in various menstrua at -87 C for 4 hours and slow thawing at 4 C for 12 hours.

A comparison of the two different buffer solutions at a similar pH indicates the greater effect of the buffer ion than the pH itself.

Because earlier data (Figure 1) showed that a relationship existed between the proportion of survivors and the concentration of leakage products and that the nature of the suspending medium also was involved, studies were conducted to determine whether any relationship existed between survival and concentration in these various menstrua. Moreover, since a regulatory mechanism seems to be involved and degradation of nucleic acids occurs, the various suspending menstrua were analyzed for the concentration of pentose. The suspending liquids in which high percentages of survivors were obtained would be expected to show low concentrations of pentose, and vice versa. The results are shown in Figure 3.

The data presented here (Figures 2 and 3) for suspensions of *E. coli* frozen at -87 C in a variety of menstrua strongly suggests a relationship between death and RNA degradation. While the cells that were suspended in magnesium solution did not show the greatest percentage of survivors, the concentration of pentose was low. Magnesium , which is known to stabilize isolated ribosomes, decreased the amount of pentose; whereas, citrate, which

(1) 0.5M phosphate buffer, pH 5.3
(2) 15% glycerol
(3) 0.2M magnesium acetate
(4) 0.02M magnesium acetate
(5) 0.002M magnesium acetate
(6) distilled water
(7) 0.02M sodium acetate
(8) 0.2M citrate buffer, pH 5.0
(9) 0.2M sodium acetate
(10) 0.85% sodium chloride solution

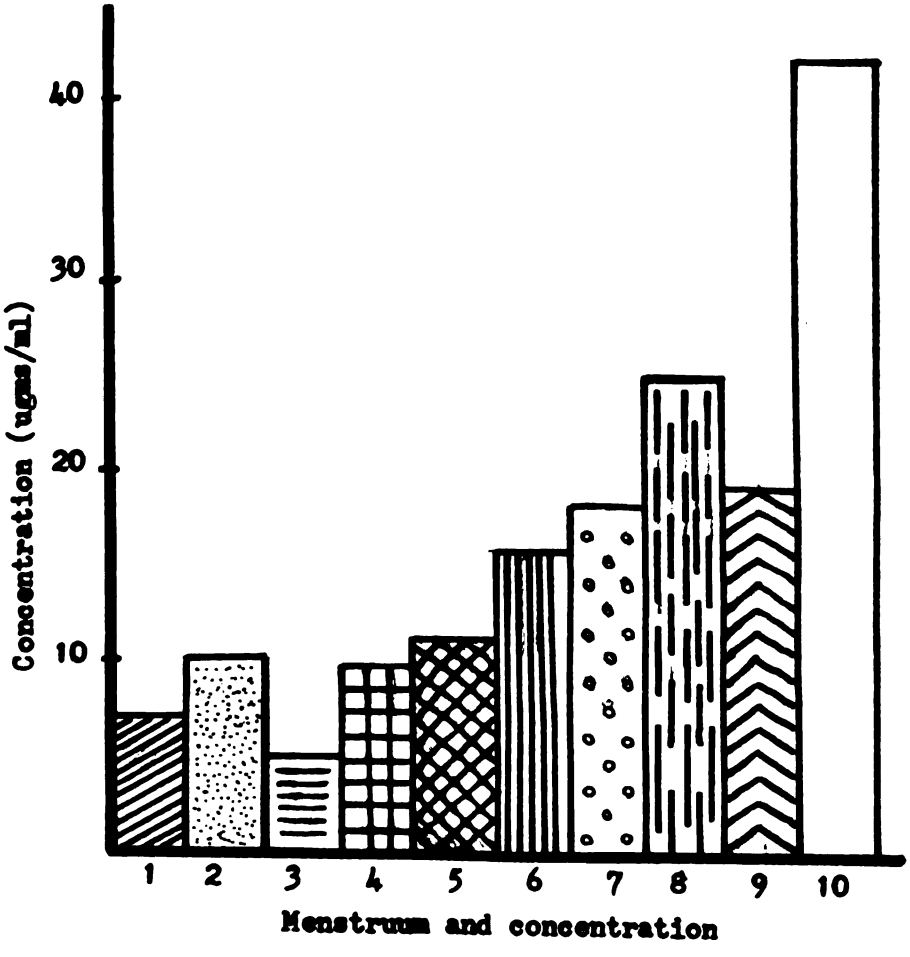

Fig. 3. Concentration of pentose in extract obtained from Escherichia coli subjected to freezing in various menstrua at -87 C for 4 hours and slow thawing at 4 C for 12 hours.

accelerates ribosomal breakdown (Chao, 1957; Wade, 1961), increased the amount.

Physiological saline solution provided the least recovery in viable numbers while distilled water and dilute solutions of sodium acetate provided only moderate recoveries. Because physiological saline solution (0.85% NaCl) produced the highest concentration of leakage products, it was selected in this study where maximum yield of extracts was desired.

Effects of thawing procedure on recovery

Since the cell suspensions were subjected to a rapid freeze and slow thaw, the concentration change in the solute would be rapid and consequently would manifest no adverse effects. This would suggest, then, that the thawing process could exert detrimental effects. A slow thaw would subject the cells to longer exposure to the concentrated solute than would a rapid thaw. Suspensions of bacteria from the same population were frozen at the same temperature; duplicate samples were removed at various intervals of time and subjected to a rapid and a slow thaw. Cell suspensions from the same population stored at 4 C were used as controls. The effects of the storage time and the type of thawing are given

in Figure 4.

These data indicate that the most drastic reduction in numbers occurs during the early stages of freezing. It is thought that this reduction is due to the decrease in temperature and the period of time during which the organisms are exposed to the critical temperatures in the range of -10 to -30 C.

While the actual freezing process appears to be responsible for the decrease in survivors, the thawing process can exert effects as well. These results would support the notion that increased concentration of solute influences survival, as these effects are markedly manifest during the thawing process.

The effects of thawing are also shown in the differences in concentration of leakage products resulting from these procedures. Cell extract was obtained from a suspension of cells that was divided into two equal volumes. After identical freezing procedures, one volume of cell suspension was fast-thawed and the other slow-thawed. Slow thawing required approximately 30-45 minutes at room temperature. The cells were removed by centrifugation, the extract diluted 1:5 with sterile 0.85% saline solution and an absorption spectrum obtained. The

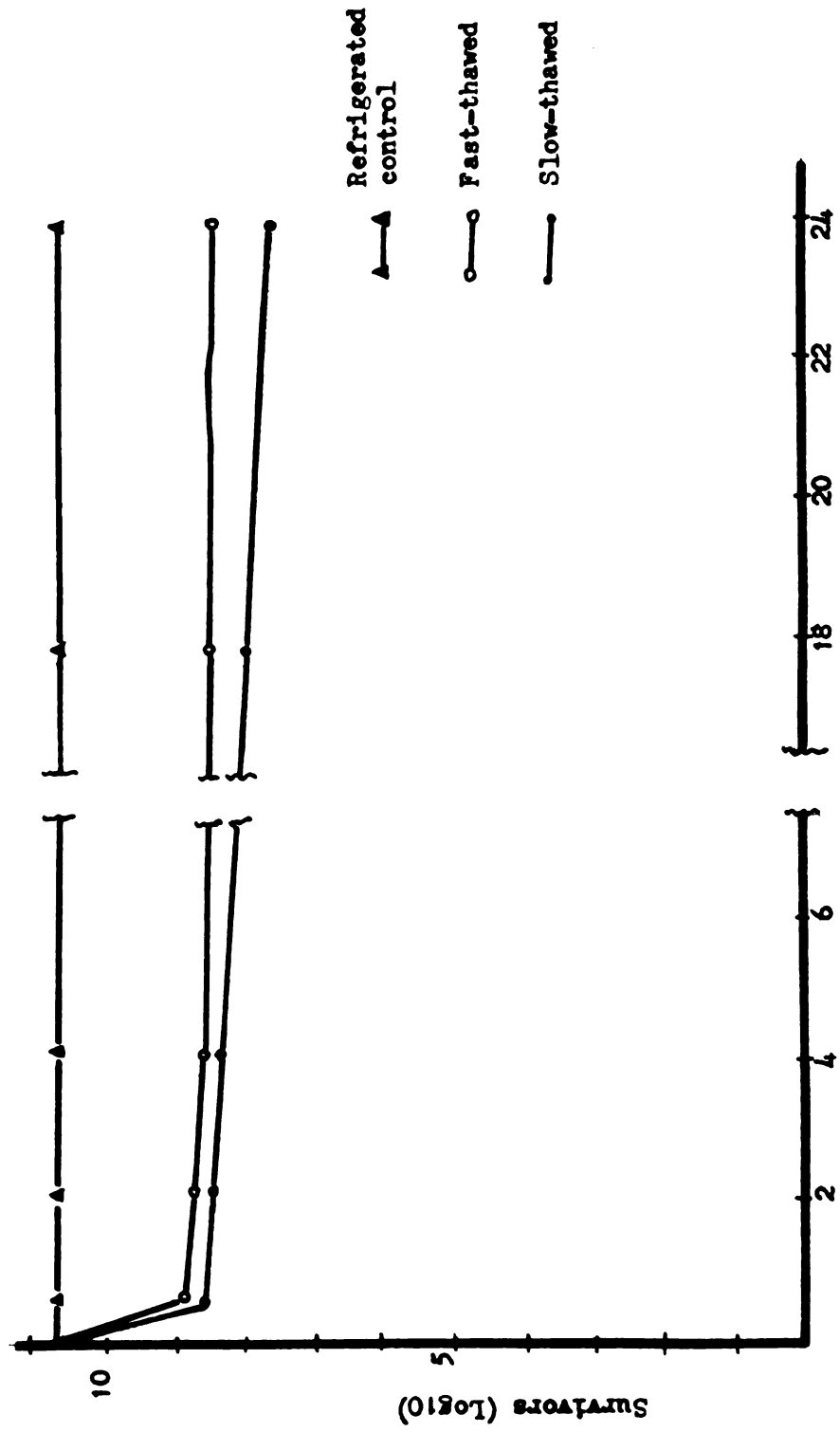

Fig. 4. A comparison of the number of survivors at various time intervals during fast-thawing and slow-thawing of Escherichia coli frozen at -87 C.

results, shown in Figure 5, indicate that the increased reduction in number, as effected by the thawing process, is accompanied by an increase in concentration of the leakage products. This, then, would support the theory that leakage is a result of increased permeability due to the increased concentration of the solute during the freezing process but the effects are more pronounced during thawing.

These data demonstrate that the loss in viable numbers during freezing is a consequence of the nature of the suspending liquid; that the greatest loss of numbers is obtained during the early stages of the freezing process, presumably during the period in which the organisms are exposed to the critical temperatures from -10 to -30 C; that the rate or type of thawing influences survival. This decrease in viable numbers is accompanied by the release of cell constituents into the medium and the concentration of these leakage products is in direct proportion to the number of survivors. The leakage of the cell constituents is apparently due to the increased permeability of the cytoplasmic membrane brought about by the increase in concentration of solute during freezing.

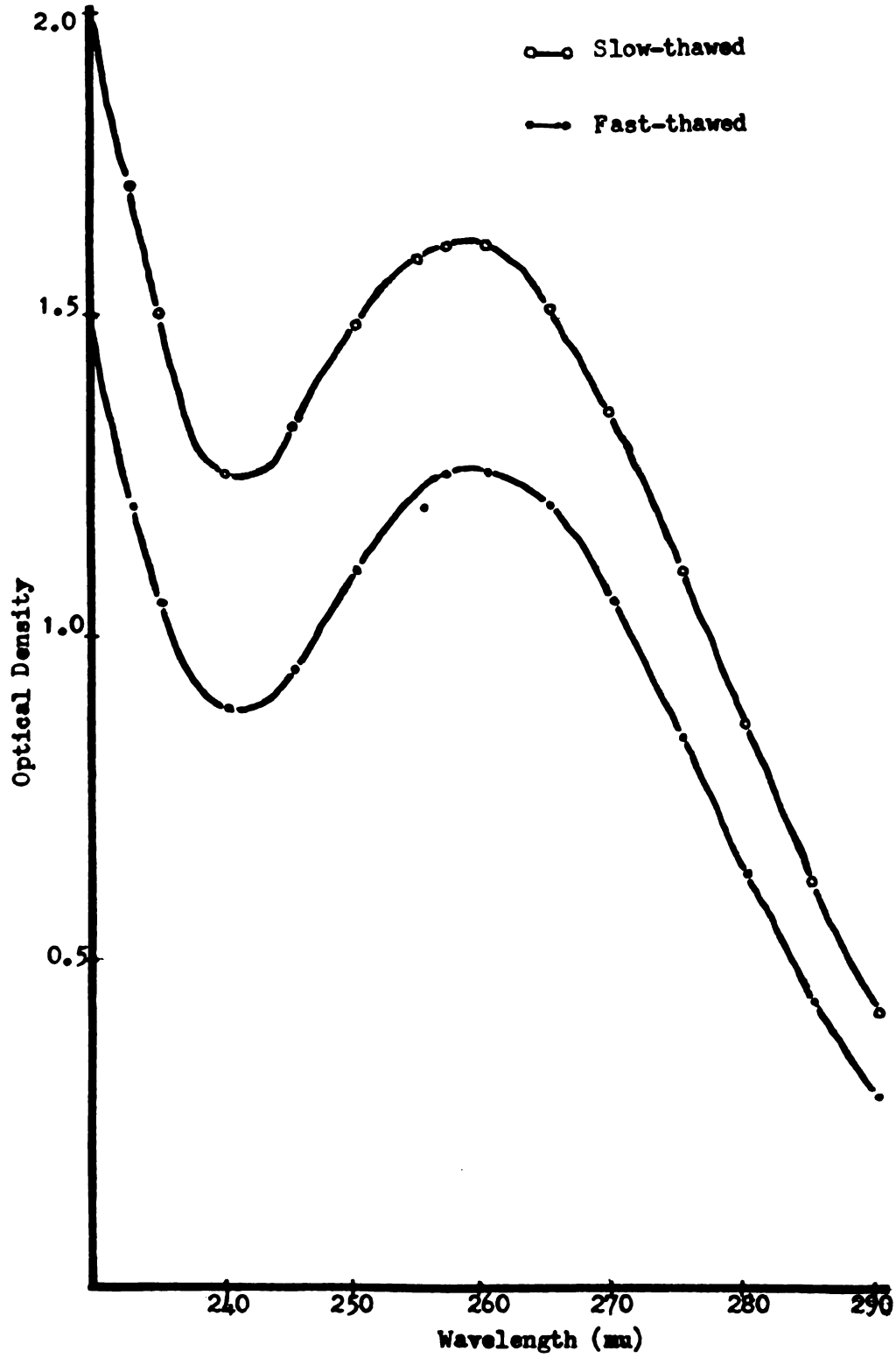

Fig. 5. Absorption sprctra of cell extracts obtained from frozen suspensions of Escherichia coli subjected to fast- and slow-thawing procedures (1:5 dilution)

Characterization of Cell Extract

Previous work by other investigators showed that leakage products exhibited at least two properties: when added to suspensions of microorganisms before stress, it exhibited protective properties; when added to suspensions of microorganisms after stress, it exhibited restorative properties. These properties were determined by increased responses of the cells in suspension. Treatment of the extract by various agents altered these properties but the nature of the active component was not ascertained.

Physical-chemical methods were used in this study to characterize the extract and correlate these data with metabolic responses of the cell suspensions.

Effect of pervaporation on the absorption spectrum of cell extract

Ten milliliters of cell extract, obtained from freezing and slow thawing a concentrated suspension of *E. coli*, were pervaporated according to the previously described method. A 1:10 dilution of the same extract was made with physiological saline solution and the absorption spectra of both made. Figure 6 shows a comparison of both spectra. From this comparison, the absence of an absorption peak in the region from 250-260 mu can be seen in the

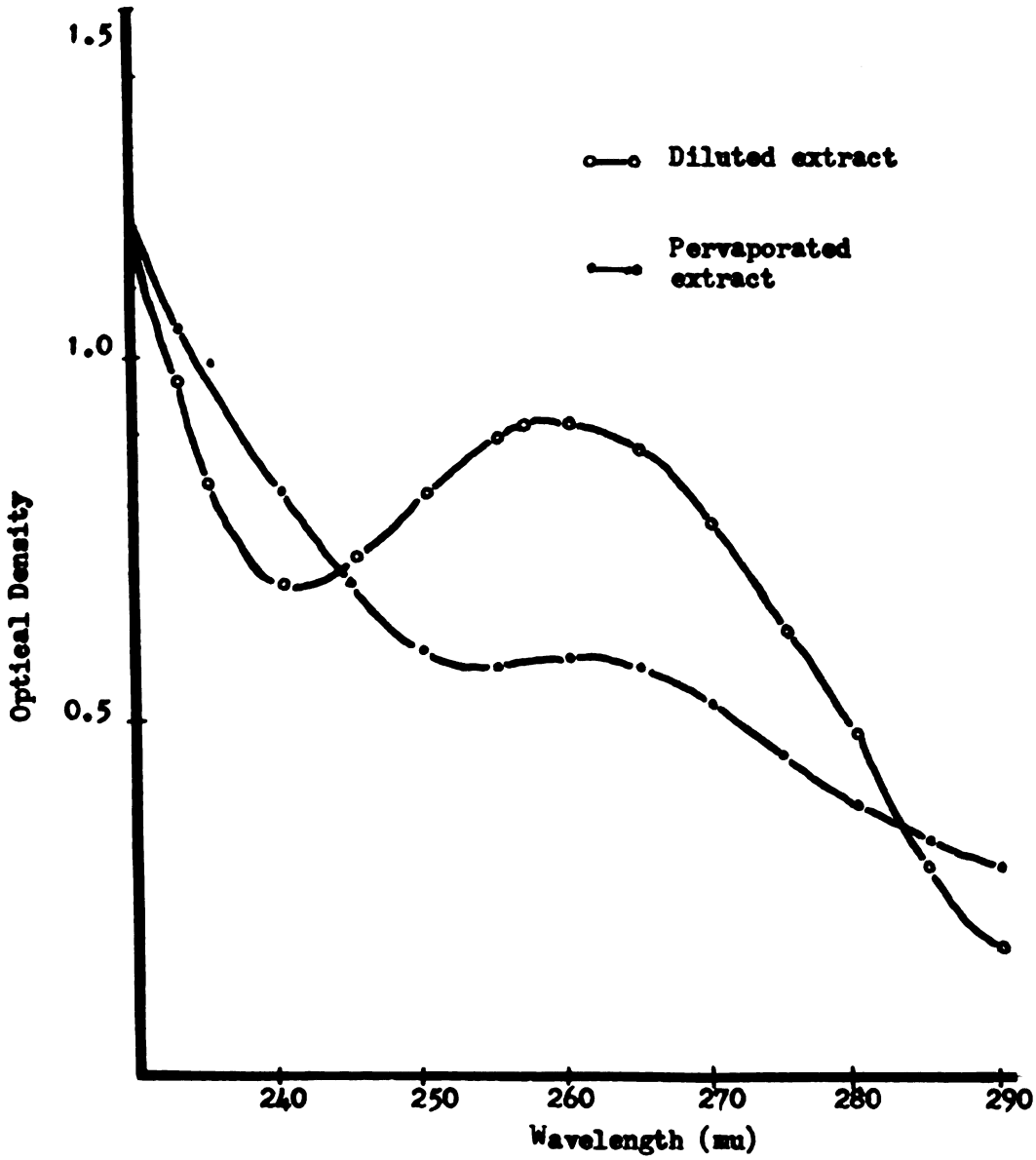

Fig. 6. A comparison of absorption spectra of diluted and pervaporated samples of cell extract obtained from frozen suspensions of Escherichia coli.

curve for the pervaporated extract.

Effect of heating on the absorption spectrum of cell extract

A 1:5 dilution of cell extract was placed in sterile 20x150 mm test tubes and heated in the previously described manner. A similar dilution (unheated) acted as a control. An absorption spectrum of both was plotted and the results given in Figure 7. From inspection of the plots, the presence of an absorption peak in the region from 250-270 mu can be seen in both the heated and unheated extracts.

In comparing the curves for both extracts, it is of passing interest to note that the only region that showed any decrease in absorption after heating was from 230-245 mu.

Effect of treatment with Norit on the absorption spectrum of cell extract

Ten milliliters of extract was treated with Norit according the the method described earlier. After removal of the charcoal, the absorption spectrum of the filtrate was determined. The results of this analysis are presented in Figure 8. From this plot, the absence of the absorption peak in the region from 250-270 mu can be seen.

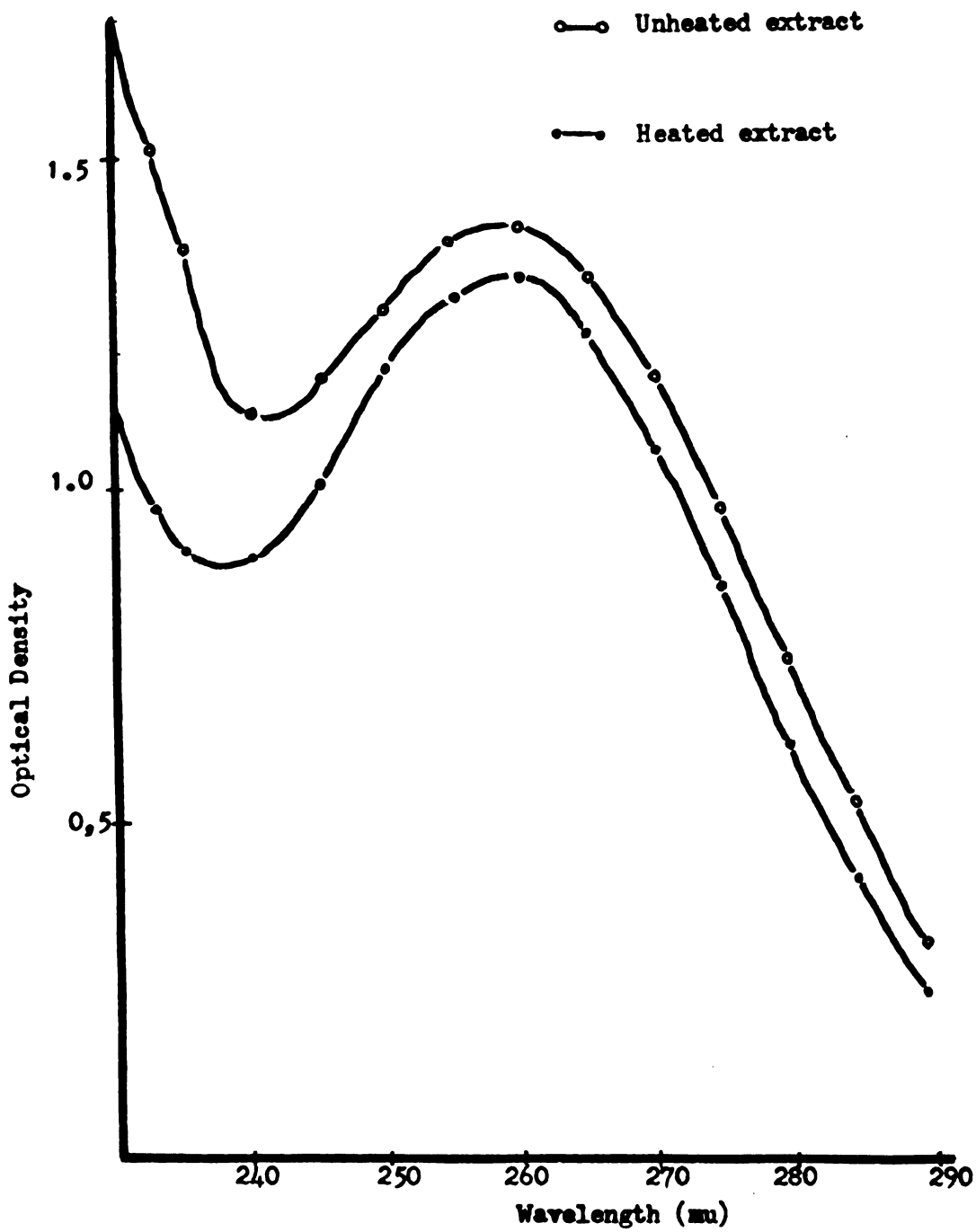

Fig. 7. A comparison of absorption spectra of heated and unheated samples of cell extract obtained from frozen suspensions of Escherichia coli.

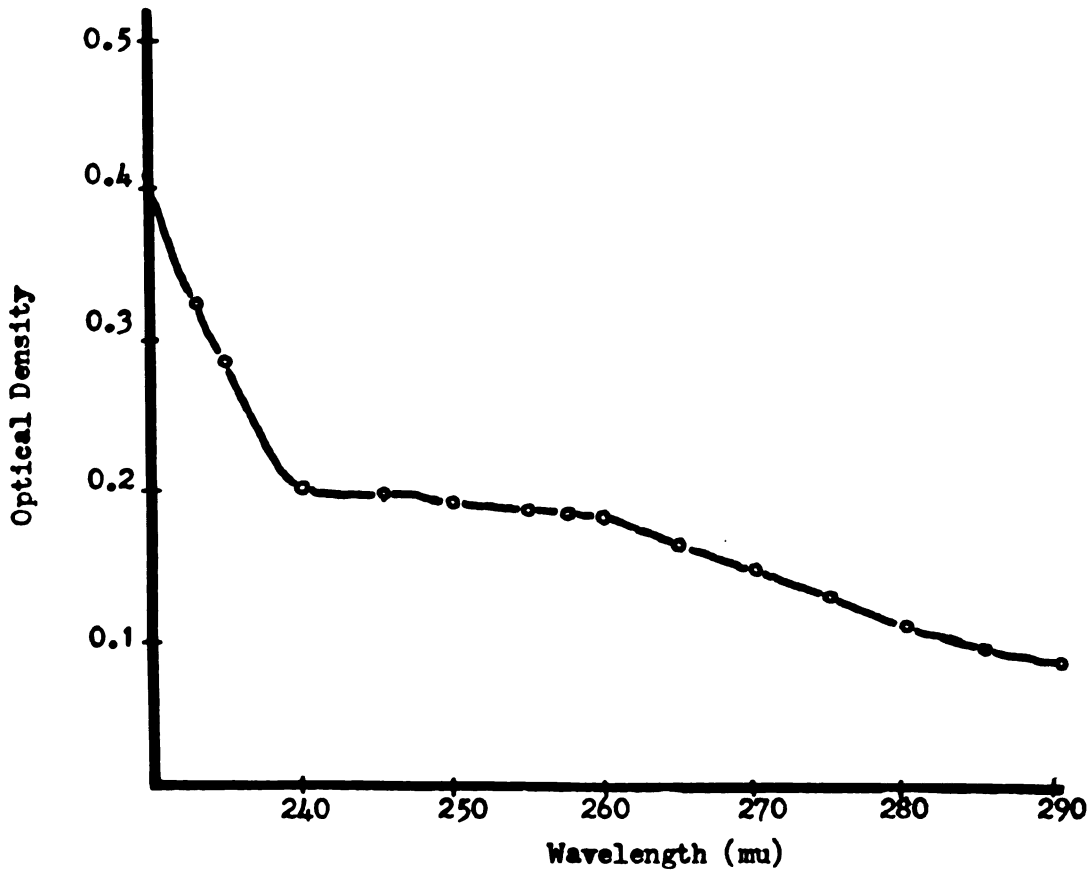

Fig. 8. Absorption spectrum of cell extract obtained from frozen suspensions of Escherichia coli after treatment with and removal of Norit.

Elution of adsorbed material from Norit

The Norit removed after treating the cell extract was washed with a mixture containing absolute ethyl alcohol-concentrated ammonium hydroxide-distilled water (25:1:24 v/v). No effort was made to keep the volume of the eluting mixture equal to the volume of the extract that was treated. The absorption spectrum of the eluted material (Figure 9) shows the characteristic absorption peak in the region from 250-270 mu. The absence of this peak in the adsorbed extract (Figure 8) and its subsequent recovery from the Norit indicates that this material was removed.

The eluate containing the ultraviolet absorbing material was evaporated to dryness with moderate heating. A crystalline residue was obtained that was freely soluble in distilled water, saline solution, acetone, HCl, and NaOH. A portion of the crystalline material was dissolved in 0.1N HCl, pH 1.0 and in 0.01N NaOH, pH 12. Volumes of material were adjusted until the absorption at 300 mu was about the same. An absorption spectrum for each solution was obtained and presented in Figure 10. Maximum absorption in acid solution was at 257 mu and in basic solution at 260 mu. The base ratios of absorbance at 250:260 and 280:260 mu were 0.955

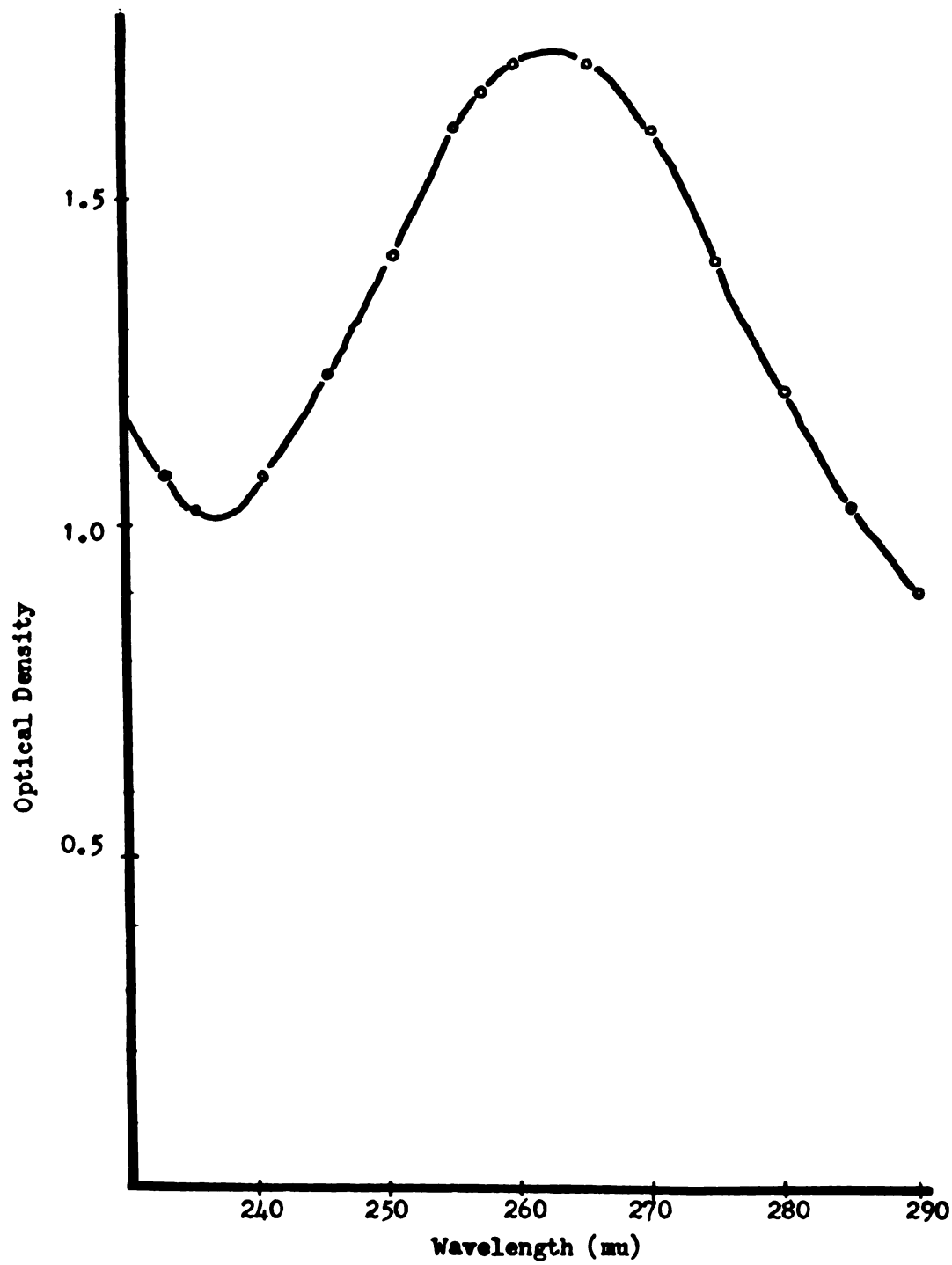

Fig. 9. Absorption spectrum of material adsorbed from cell extract obtained from frozen suspensions of Escherichia coli after adsorption with and elution from Norit.

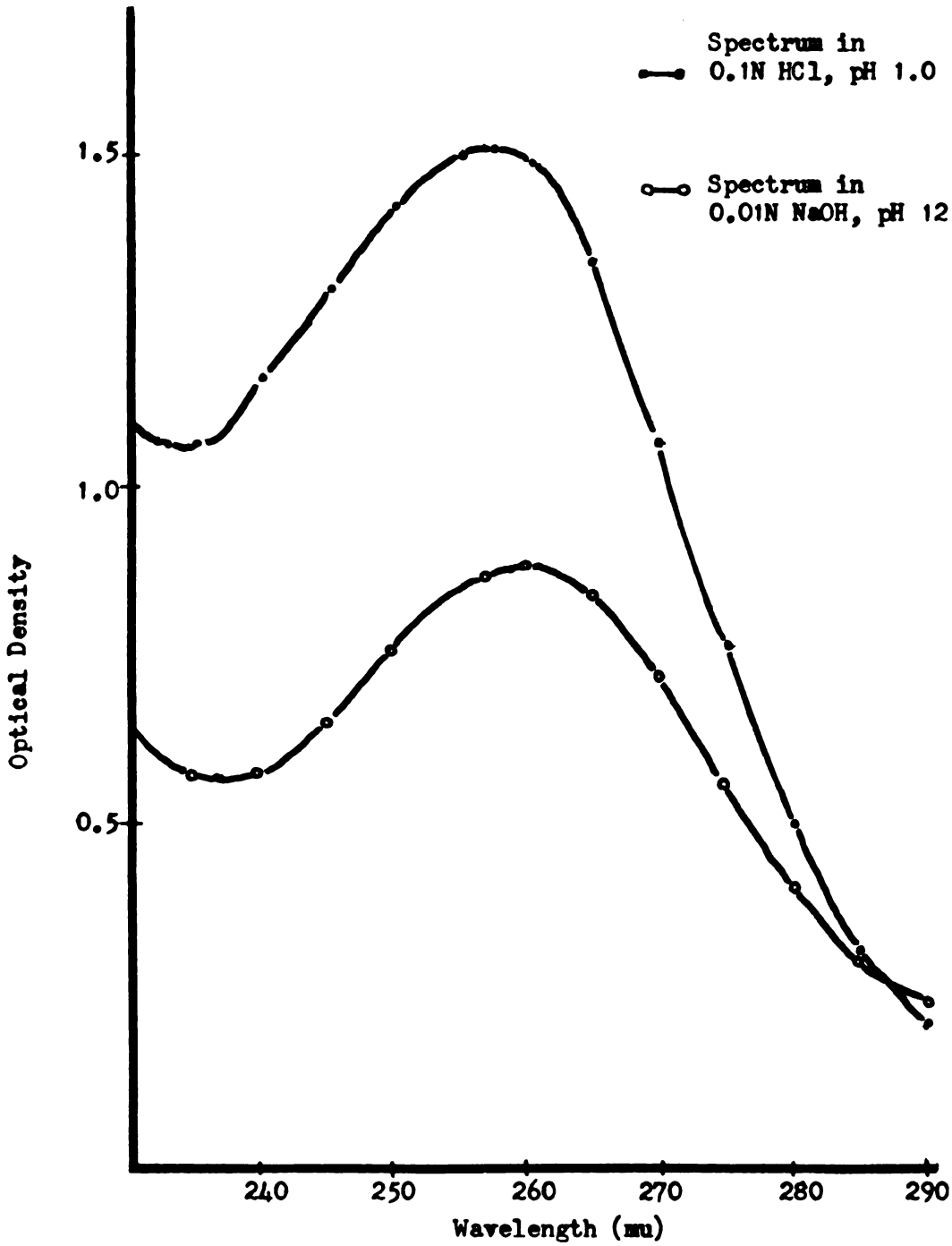

Fig. 10. A comparison of absorption spectra of ultraviolet absorbing material Norit-adsorbed from cell extract obtained from frozen suspensions of Escherichia coli and suspended in acid and basic solutions.

and 0.34 respectively in acid solution and 0.885 and 0.466 respectively in basic solution. Absorption maxima and base ratios were compared with those given in a chart for nucleic acid derivatives prepared by Calbiochem Corporation, Los Angeles, California. The absorption maximum in acid and base coincided with those presented for nucleosides and nucleotides of the purine adenine. However, no agreement exists between the base ratios obtained for the crystalline material and those presented for compounds containing adenine.

The base ratios of the extract at 250:260 and 280:260 mu were 0.90 and 0.50 respectively and are in accord with those presented by other investigators for their extracts. No base ratios for extracts obtained from frozen suspensions of Aerobacter aerogenes were available or presented. To determine these values an extract was prepared using concentrated suspensions of Aerobacter aerogenes. The absorption spectrum for this extract is given in Figure 11. Maximum absorption occurred at 260 mu and base ratios at 250:260 and 280:260 mu were 0.94 and 0.64 respectively. Agreement exists only with the absorption maximum; discrepancies in values for base ratios might be attributed to the absorption of other leakage products or else to the

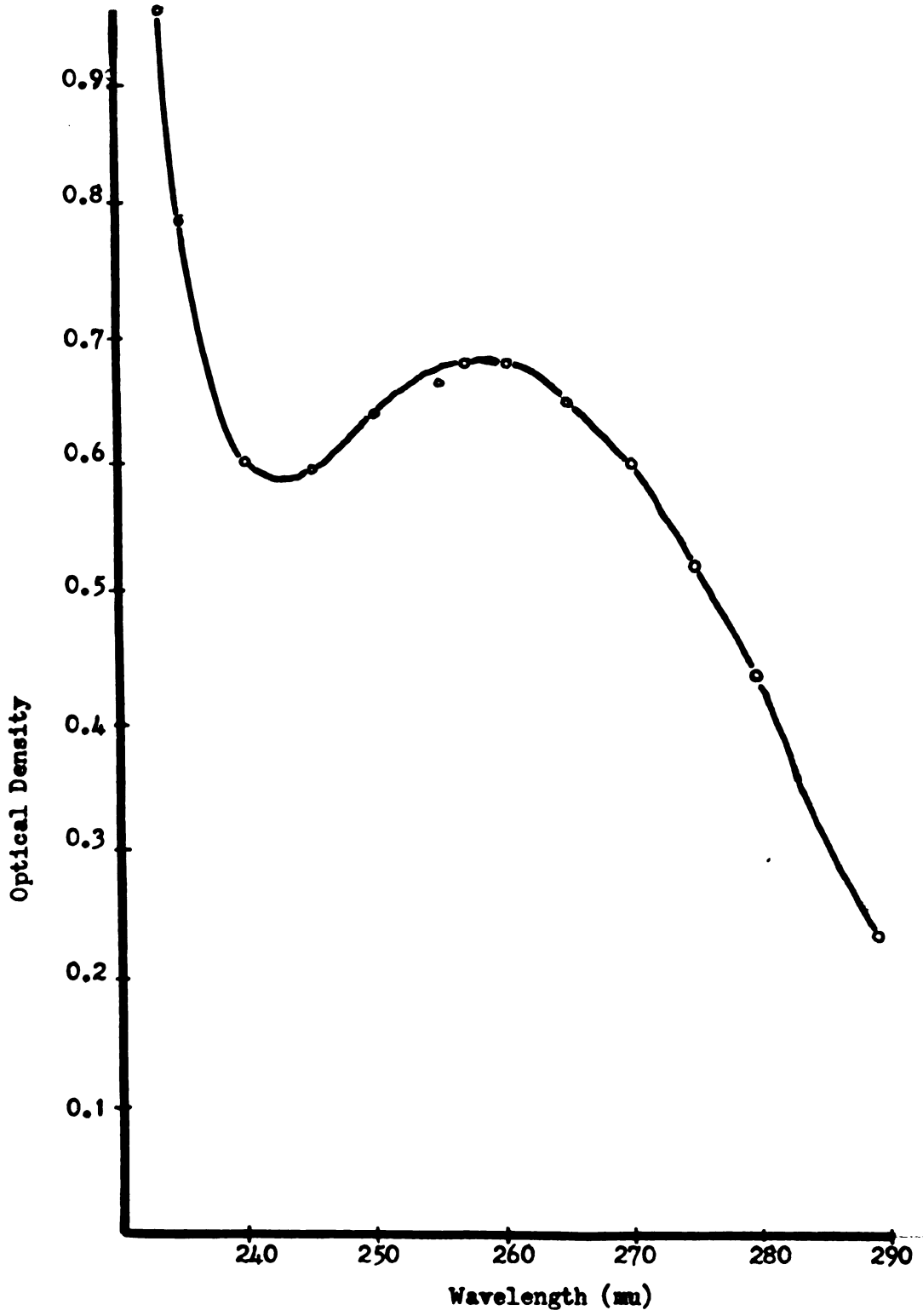

Fig. 11. Absorption spectrum of cell extract obtained from frozen suspensions of Aerobacter aerogenes (1:5 dilution)

fact that the ultraviolet absorbing material in this extract is different.

The loss of the U-V absorbing material upon pervaporation suggests a low molecular weight compound that would discount the possibility of it being RNA.

Fractionation of cell extract

Since the activity of the cell extract appears to be related with the ultraviolet absorbing material, fractionation was performed using the procedure outlined in Manometric Techniques. Four fractions were obtained and the absorption spectrum of each fraction determined (Figure 12).

The presence of the characteristic peak absorption in the region of 250-270 mu in the TCA-soluble fraction gives added evidence that the U-V absorbing material is not RNA.

The presence of polysaccharide material in the extract may exhibit colloidal properties and prevent the precipitation of RNA upon the addition of TCA. If this were the case, then it would seem likely that the addition of barium would result in its precipitation. The absence of any absorption peak in the barium-insoluble fraction appears unlikely that RNA in any form is present.

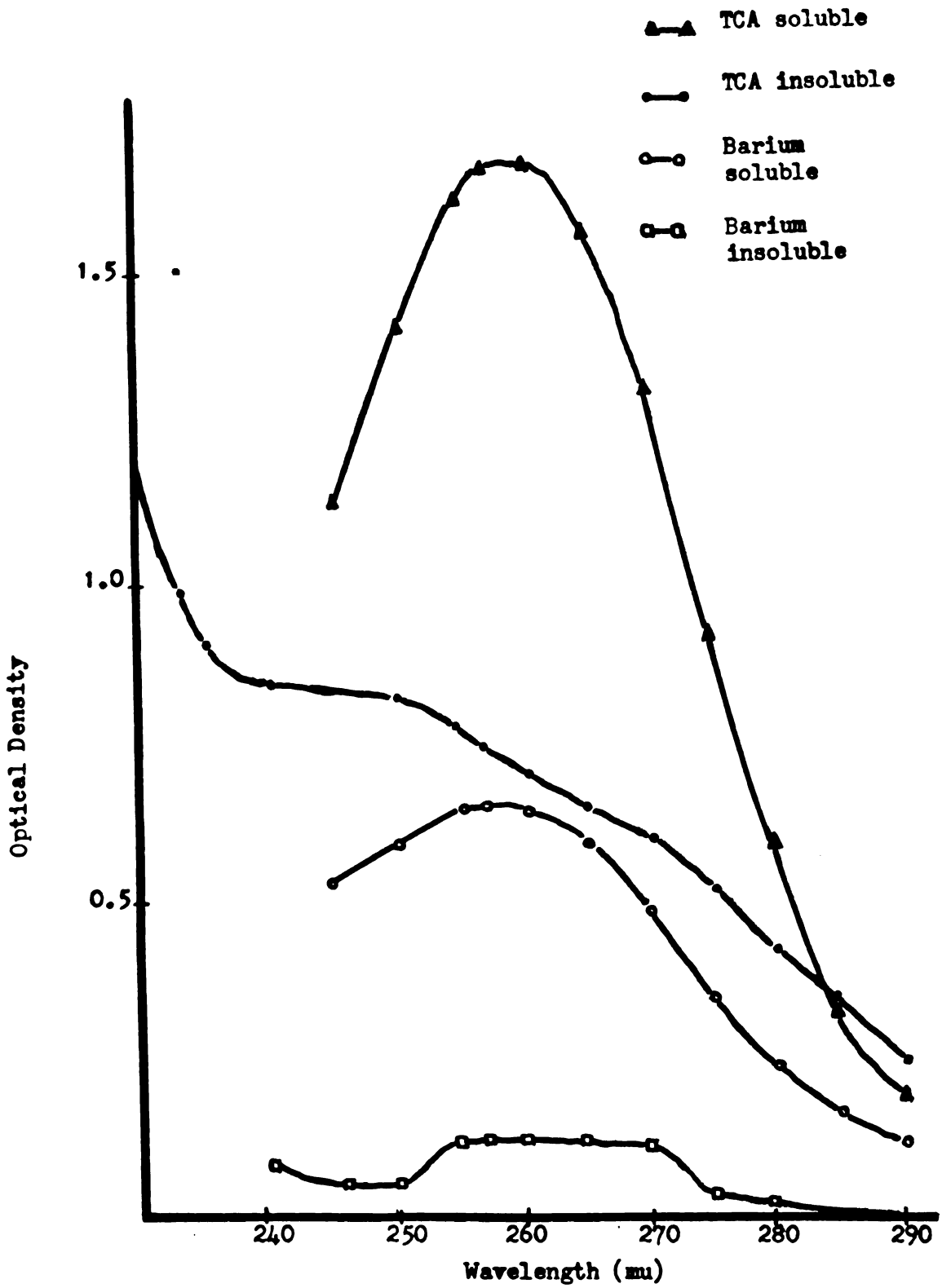

Fig. 12. Absorption spectra of various fractions resulting from the fractionation of cell extract obtained from frozen suspensions of Escherichia coli

The U-V material is present in the barium-soluble fraction. Maximum absorption occurs at 257 mu with ratios at 250:260 and 280:260 mu at 0.91 and 0.36 respectively. Since the material was in an acid solution, these ratios most nearly approximate those for adenosine monophosphate (AMP). Colorimetric analysis for pentose and inorganic phosphate gave molar ratios of 0.04:0.033 micromoles/ml which is approximately 1:1. There was no increase in the concentration of the phosphate after acid hydrolysis which indicates a monophosphate. A relationship between color development in the orcinol test and the position of the phosphate linkage has been noted. This method can be used for differentiating between a 5' linkage and a 3' linkage. A 5' linkage gives maximum color development in a shorter period of time than the 3' linkage. Maximum color development in the pentose determination was attained in 15 minutes, which would indicate a 5' linkage of the phosphate.

From the data presented in Manometric Techniques the compound present in the barium-soluble fraction that absorbs ultraviolet light is AMP. On the assumption that this was the compound present in the barium-soluble fraction after fractionation, the concentration was estimated from the spectrophoto-

metric data. From the optical density at the maximum absorption and the extinction coefficient (14,900 $M^{-1}cm^{-1}$), a value of 0.042 micromoles/ml was obtained. This value coincided with the concentrations of pentose and phosphate for this fraction and gives a ratio of 1:1:1 for base:sugar:phosphate-- a model that fits ATP.

Effect of ribonuclease on ultraviolet absorbing material

Since RNA also provides a model from which a ratio of 1:1:1 for base:sugar:phosphate can be obtained, the ultraviolet absorbing material was treated with bovine pancreatic ribonuclease. An increase in optical density at 260 mu was used as a measurement of ribonuclease activity. No increase was detected which would indicate that the ultraviolet absorbing material is not RNA.

Electrophoresis of cell extract

Present in the extract with the ultraviolet absorbing material was a substance that reacted with the Folin reagent. The substance was considered to be protein, but the results of the treatment of the cell extract with cold 10% TCA (Figure 10) showed no decrease in absorption in the region of 280 mu which is characteristic for protein. Also, the

precipitate showed no peak in this region when it was resuspended and the spectrum determined. After adsorbing the ultraviolet material from the extract with Norit, no peak was obtained at 280 mu. Extracts obtained from E. coli and Aerobacter aerogenes were subjected to electrophoretic separation to determine if any differences existed between them. The results are diagrammed in Figures 13a and 13b.

More bands could be detected in the extract obtained from Aerobacter and the pattern differed also. These differences could possibly account for the discrepancies noted in the ratios at 250:260 and 280:260 mu for the absorption spectra of the extracts. Pervaporation of the extract yielded material that gave the same pattern which would indicate that this material does not seem to be the active substance.

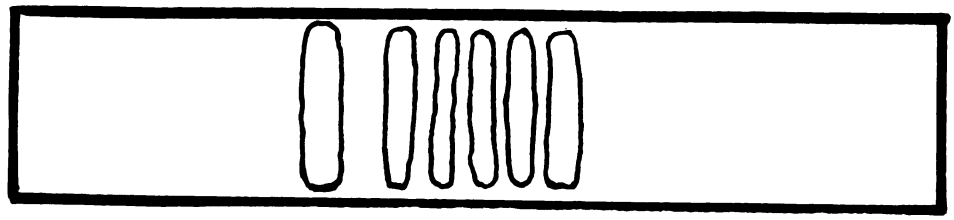

Fig. 13a. Electrophoretogram of cell extract obtained from frozen suspensions of Escherichia coli

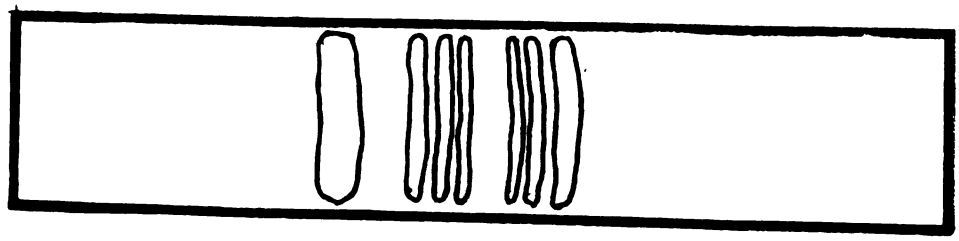

Fig. 13b. Electrophoretogram of cell extract obtained from frozen suspensions of Aerobacter aerogenes.

DISCUSSION

The injury that results when microorganisms are frozen and thawed is manifested by losses in viable number and the leakage of intracellular constituents. Such results can be attributable to two causes: lysis or rupturing of the cell wall, which gives form and rigidity to the cell; or, some change in the cell membrane, the structure responsible for maintaining the integrity of the internal constituents. Direct observation of frozen cells under oil immersion and electronmicrographs of frozen and unfrozen cells (Meyhnell, 1958) present indirect evidence eliminating lysis as a factor.

The absence of any detectable deoxypentose in the cell extract would also indicate that lysis did not occur. Cells ruptured by sonic vibration showed the presence of this carbohydrate which is attributed to DNA. If frozen cells are lysed or ruptured, then, they too should liberate DNA. None was detected in this study. As a result, such results would refute any theory of death as a result of crushing.

The effects of freezing apparently lie in some alteration of the cell membrane. The cell membrane not only prevents leakage of the cytoplasmic constituents into the environment but is also concerned

with the transport of nutrients into the cell. Any agent or stress that damages this semi-permeable membrane by altering its physico-chemical structure will cause disorganization of cellular function. When its selective properties are lost, components leak out of the cell resulting in injury or death.

Evidence to support this view can be obtained from the data presented on freezing. A relationship has been established between the viable number of cells recovered after freezing and the loss of internal diffusible cellular constituents. This effect is considered by Mitchell and Moyle (1956) to be due to differences in the internal osmotic pressure. If this were true, then distilled water should have exerted the least amount of stress and given the greatest amount of recovery. The data obtained in this study indicate that distilled water provided only moderate protection which would discount this theory related to osmotic pressure. These observations are also in conflict with those recorded by Harrison (1956) and Clement (1961) who found distilled water a relatively innocuous environment for freezing. Osmotic effects can also be eliminated on the basis of recovery after the organisms were frozen in other menstrua. Higher molarities of the solute should have provided the

highest death rates if osmotic pressure were responsible. Such an effect was noted only with sodium acetate.

When the cells were suspended in the various menstrua, a negligible period of time (approximately 5 minutes) elapsed before freezing. This would eliminate any possibility of incubation in the solute or penetration of the agent. Under such conditions, these results are incompatible with a view that any protection provided during freezing requires (a) penetration of the agent and (b) osmotic dehydration. Postgate and Hunter (1961) obtained equally effective protection from substances that did and did not penetrate.

The various responses that were obtained in this study must be attributed to the effect that the particular solute has on the microorganism.

Moderately high recoveries were obtained with glycerol. The protective action of glycerol was demonstrated to be compatible with the results obtained by other investigators. The protective action is attributed to the ability of the glycerol to stabilize permeability.

The losses in number in menstrua that should have provided protection might be due to the washing process in preparing the cells for suspension. In this

study, saline solutions were used. Washing with saline solution has been reported to desorb magnesium ions from the cells (Strange and Shon, 1964) which increased sensitivity to thermal stress. The addition of magnesium reversed these effects.

While the cells that were suspended in magnesium solutions did not show the greatest percentage of survivors, the concentration of pentose was low. The activity of magnesium lies in its stabilization of the ribosome and ribonucleo-protein (Lederberg, 1956; Weibull, 1953). When these ions are replaced or removed by monovalent cations, the ribonucleic particles undergo a series of dissociations-- first reversible, then irreversible (Elson and Tal, 1959). This process could occur either during the washing process or in the final suspension. The degree of dissociation could account for the ability of some agents to increase the recovery of survivors-- providing the essential nutrients to aid recovery are present.

Such an effect on the stability of the ribosome can not only be seen with magnesium, which decreased the amount of pentose, but also with citrate, which accelerates ribosomal breakdown and increased the amount of pentose. Similar effects with citrate were reported by Chao (1957) and Wade (1961).

How can the extreme toxicity of sodium be explained? First of all, the cell membrane has been found to be permeable to the sodium ion (Mitchell and Moyle, 1959). This would explain the breakdown of the ribonucleoprotein and the leakage of the intracellular products. Elson (1958) obtained similar results. The addition of sodium chloride to isolated ribonucleoprotein affected a separation of the molecule. Secondly, sodium is important in water metabolism of cells. It controls the movement of water and sodium intake effects or controls hydration. Replacement of lost electrolytes during the washing procedure with saline solution leads to migration into the cells which would produce profound alteration.

While these hypotheses might explain the effects of various salts on viability, they do not explain why there should be reduction in viable numbers when cells are frozen on membranes in the absence of any suspending medium. The cells were reported to behave similarly to cells frozen in a menstruum (Bretz, 1961) in that there was a nutritional demand. However, no data were available concerning leakage.

The emphasis on the behavior of cells that have been exposed to sub-zero temperatures has been on the freezing process. Truly, it should, but the data presented indicates that thawing plays a role in this

loss in numbers and leakage. Unfortunately, there is no way to determine whether the thawing process is in essence the factor responsible for the loss in viability. The evidence at least suggests that it is partially involved, even if it is only because it increases the amount of time during which the cells are in suspension.

During the freezing process, the most drastic reduction in numbers of viable cells occurs within a half-hour after solidification. In the time study, the cell suspensions were frozen and samples removed for thawing and assay after various time intervals. The suspensions were observed during freezing and samples removed immediately upon solidification. This point was taken as the zero-time for the study. The results showed that there was no decrease in viable numbers and the type of thaw had no additional effect. It would seem, then, that the actual process of ice formation is not the deleterious agent. The largest reduction in numbers occurred within 30 minutes after solidification with no significant reduction afterwards during storage. It appears that the reduction occurs during the time that the microorganisms were exposed to the critical temperature between -10 and -30 C. Why this temperature range is critical has not been ascertained. In as much as

this range is considered critical for most microorganisms studied, and that external ice has already formed, it would not seem unlikely that this is the range in which the internal solutes of the cell solidify. The internal constituents do comprise a complex mixture of material which surely must have a freezing point that is lower than most of the substances used as suspending menstrua.

The formation of intracellular ice would explain the behavior of cells frozen on membranes. Internal freezing could cause the alterations in cell membranes since these would be in direct contact with the frozen material, whereas they would not in the case of external ice formation. The formation of intracellular ice would not occur upon initial freezing of the suspending liquid which would account for the high proportion of survivors that were recovered during the first formation of ice.

The data presented in this study on the effects of freezing and the responses given by the cells in suspension strongly suggest that the cause of the phenomena is an increase in cell permeability. Substances that stabilize permeability provided protection while those that did not provided a decrease in recovery and an increase in the amount of pentose released. Although there is this release of material

from the cells, it does not necessarily follow that death occurs. Rotman (1958) found that cells can lose as much as 80% of their RNA and still remain viable.

The leakage material present in the suspending medium has been reported to possess protective and restorative properties. Protection by the extract has been demonstrated by Strange and Shon (1964) with increased resistance of cells that were exposed to heating, and to a lesser extent by Postgate and Hunter (1961) who obtained some protection from freezing when the extract was added to the suspending medium. Bretz and Ambrosini (1963) obtained a protective factor for the increased survival of E. coli that were frozen and thawed. Survival was correlated with the carbohydrate fraction of the extract.

Restoration, in the form of stimulation of activity, has been demonstrated by DeLamater et. al. (1958) with extract obtained by leaching cells in phosphate buffer solution. The material stimulated oxygen uptake in the presence of glucose. Similar results were obtained by Roszman (1963) who obtained increased oxygen uptake when extract was added to suspensions of frozen and thawed cells. Mehler and Hartsell (1963) isolated a growth stimulating substance from E. coli that they termed

"Factor S." The stimulating activity of the extract seems to be related to the ultraviolet absorbing material and spectrophotometric analyses indicates that the presence of a peak in the region from 250-270 mu corresponds with activity. The results obtained in this study are in direct agreement with those obtained in this study by Rossman. When the cell extract is treated by the same technique he used, the presence or absence of absorption peaks coincided with his reported changes in activity of the cells.

The characteristics of the U-V absorbing material obtained in this study differ from those reported by other investigators. Postgate and Hunter (1962) isolated U-V material from starved Aerobacter aerogenes with an absorption peak near 260 mu. The compound was considered to be adenosine triphosphate (ATP). However, this material could not account for all of the U-V absorption. Strange and Shon (1964) obtained a compound absorbing maximally at 255 mu from heated Aerobacter aerogenes that possessed characteristics of deaminated hypoxanthine. DeLamater et. al. (1958) leached a substance from suspensions of Bacillus megaterium that absorbed maximally at 258 mu. At pH 7.0 base ratios at 250:260 and 280:260 mu were 0.89 and 0.50 respectively. No loss of absorption

occurred upon dialysis and hydrolysis and chromatography yielded the four nucleotides present in RNA. Robrish and Marr (1957) obtained extracts from osmotically disrupted Azotobacter that had strong absorption at 258 mu which was presumed to be RNA. A substance was detected in frozen suspensions of E. coli by Lindegerg and Lode (1963) that had maximum absorption near 260 mu. The base ratios of the compound at 250:260 and 280:260 mu were 0.89 and 0.49 respectively.

The base ratios at 250:260 and 280:260 of the U-V material obtained from E. coli in this study were 0.90 and 0.50 respectively. While these are in agreement with those obtained by DeLamater for Bacillus megaterium and Lindeberg for E. coli, they do not agree with those obtained for material present in extracts from Acrobacter that were used in this study. Agreement seems to be only with the maximum absorption wavelength.

Rossman found that dialysis resulted in a loss of the stimulating activity of the extract. The spectrophotometric results of pervaporated extracts in this study showed the absence of any absorption peak in the U-V region. These data are in opposition with the results of DeLamater who reported no loss of absorption or activity upon dialysis of the extract.

It appears then that the U-V material is not the same. Since DeLamater obtained the four nucleotides present in RNA upon hydrolysis, it seems unlikely that the product present in the extract obtained from E. coli that absorbs U-V is RNA.

Additional evidence against the product being RNA is found in the fractionation procedure. No U-V material was present in the TCA-insoluble fraction. Although RNA is insoluble in cold TCA, McQuillen (1950) reported an acid-soluble material absorbing at 260 mu released from E. coli when penicillin was added to growing cultures. However, the product was never identified.

The characterization of the U-V absorbing material as adenosine monophosphate (AMP) was made on the basis of its solubility in barium and the ratio of the base:sugar:phosphate. The phosphate concentration was determined by a seven-minute hydrolysis in HCl which would liberate the phosphate groups. Any increase during this hydrolysis would be attributed to the presence of di- or tri- phosphates. Since no increase was obtained, a monophosphate was concluded as being present.

The position of the phosphate group on the pentose has been shown to affect the speed of color development in the orcinol reaction. Albaum and

Umbreit (1947) used this as a method for differentiating between 5' and 3' linkages. With 3' linkages, maximum color development is attained after approximately 1 hour at 100 C, whereas with a 5' linkage maximum color development is attained after 15 minutes. No increase was evident upon continued heating of the tubes during this determination. Consequently, it was concluded that the phosphate was linked to the pentose in a 5' position. From the spectrophotometric data, concentration of pentose and phosphate, and the rate of color development, the compound was considered to be adenosine monophosphate (AMP).

Although other data suggest that RNA is not present as the U-V absorbing material, it does provide a model that fits a 1:1:1 ratio of base:sugar:phosphate. However, the results obtained with the enzyme ribonuclease strongly indicate that this is not present.

The material that is apparently considered to be protein might be peptide. Since no absorption peaks were noticed in the region of 280 mu, which is specific for proteins, it is possible that no protein is present or else the protein lacks the amino acids that give the characteristic absorption peak-- tyrosine and tryptophane. The Folin reagent

is 10-20 times more sensitive than absorption at 280 mu which could account for the detection colorimetrically but not by absorption.

It is of interest to note that the electrophoretic pattern obtained from the extracts was always the same. This would indicate that the material that leaks as a result of freezing comes from some cell structure that has a definite composition. If the leakage of the protein-like material came from indiscriminate sites, then an array of patterns would have been obtained. The ribosome would be one such cell structure that would possess a definite composition which gives further evidence that this is the structure involved.

From these data, it is concluded that the effects of freezing are due to an increased permeability of the cell membrane and the leakage of material from the cell as a result of the dissociation of some cell structure with a definite composition.

SUMMARY

Different percentages of viable cells were obtained when suspensions of microorganisms were frozen at -87 C for four hours and thawed. Survival depended upon the nature of the suspending menstruum. Cells suspended in phosphate buffer solutions (0.5M) survived better than cells suspended in 0.85% saline solution.

Crushing as a result of ice formation was eliminated as a factor since very few cells were lost upon solidification of the menstruum. The most drastic reduction in numbers occurred after 1 hour of freezing with no appreciable loss obtained during subsequent storage.

The rate of thawing frozen suspensions contributed to the decrease in viable numbers. A slow thaw was more detrimental than a fast thaw.

Death of the microorganisms was accompanied by the release of cell constituents into the suspending medium. These products consisted of a protein-like substance, pentose, and a substance that absorbed ultraviolet light. No deoxypentose could be detected and paper chromatography showed no free amino acids. Leakage of cell constituents indicated that a permeability control mechanism was affected. A corre-

lation existed between the percentage of survivors and the concentration of the leakage products in the suspending medium.

Absorption spectra of extracts obtained from frozen suspensions of microorganisms showed that the ultraviolet absorbing material had a maximum absorption at 257 mu. Additional spectra of treated extracts showed that the ultraviolet absorbing material was heat-stable, dialyzable, could be adsorbed from solution on Norit, and could be eluted from the Norit. The reported activity of the cell extract appears to be related with the ultraviolet absorbing material.

Fractionation of cell extracts yielded four fractions and absorption spectra of these fractions demonstrated that the ultraviolet absorbing material was recovered in the barium soluble fraction. The behavior of the ultraviolet absorbing material during fractionation lead to the conclusion that this material was not RNA. Later tests with ribonuclease confirmed this conclusion.

The ratio of the base:sugar:phosphate, obtained by physical and chemical means, most nearly approximated those for adenosine monophosphate. The relationship between the rate of color development of the pentose in the orcinol test and the position of the phosphate linkage indicated that the phosphate was

linked to the pentose in the 5' position.

Electrophoresis of cell extracts obtained from both E. coli and A. aerogenes presented patterns that differed in both the number and position of bands. However, the same pattern was obtained for each extract in every instance intimating that the same site in the cell was affected by freezing. The protection afforded by magnesium salts and the detrimental effect of citrate and sodium paralleled the behavior reported for isolated ribosomes.

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