1933

The effect of hypertonic sugar solutions on the thermal resistance of bacteria

Arthur Cecil Fay
Iowa State College

NOTE TO USERS

THE EFFECT OF HYPERTONIC SUGAR SOLUTIONS ON THE THERMAL RESISTANCE OF BACTERIA

BY

ARTHUR CECIL FAY

A Thesis Submitted to the Graduate Faculty
for the Degree

DOCTOR OF PHILOSOPHY

Major Subject Dairy Bacteriology

Approved:

Signature was redacted for privacy.

In charge of Major Work

Signature was redacted for privacy.

Head of Major Department

Signature was redacted for privacy.

Dean of Graduate College

IOWA STATE COLLEGE
1933

Outline

INTRODUCTION

In connection with the various studies on the bacteriology of ice cream which have been in progress at the Kansas Agricultural Experiment Station for several years, it has been observed occasionally that microorganisms exhibit an increased thermal resistance when heated in ice cream mix. Although these observations have been more or less incidental to other experiments they occurred with sufficient frequency to justify more specific investigation. Experiments involving the pasteurization of ice cream mix which previously had been inoculated showed the survival of organisms known to be incapable of withstanding similar exposures when heated in milk or broth. The inconsistency of such observations, together with the fact that the experiments usually were designed for other purposes and consequently were not properly controlled on this point, discouraged definite conclusion.

The temperature and time of exposure most commonly employed in the pasteurization of ice cream mix have been adopted from the market milk industry without question as to the universality of their application. If thermal resistance is affected by the chemical and physical forces of the environment it is entirely logical to expect a variation in the

survival of cells heated in different menstrum. The small margin of safety in the present requirements for pasteurization emphasizes the importance of evaluating any increase in thermal resistance of the microflora which may be contributed by the ingredients of ice cream.

Preliminary experiments showed quite definitely that certain strains of bacteria were capable of withstanding more severe heat treatment when suspended in solutions of high osmotic pressure. These observations suggested a possible relationship to the previous experiences with increased thermal resistance of certain bacteria in ice cream. At about the same time, the results reported by Beavens (1930) and Fabian and Coulter (1930 a) further suggested that physico-chemical forces should not be ignored in evaluating the resistance of cells to heat.

Although the primary interest in this problem centered about its possible relation to the survival of bacteria in the pasteurization of ice cream mix, relatively few of the experiments to be included in this report have involved the use of this product as the heating medium. Due to the lack of specific information on the effect of certain physical forces in ice cream on thermal resistance, the study has more or less evolved into an analysis of the fundamental rather than the practical aspects of the problems of cell destruction.

It is apparent that an analysis of the fundamental factors underlying such protective action would necessarily in-

volve a study of the mechanism of the death of the cell. A survey of the literature reveals a surprising lack of specific information on this important process. The very fact that the literature on the subject is voluminous, together with the many past and present controversies over the precise mechanism by which a cell dies, is, in itself, evidence of the lack of concrete information.

REVIEW OF LITERATURE

Protective Action

Ice cream. It has been recognized for several years that microorganisms could withstand more heat or tolerate higher concentrations of disinfectants when in the presence of certain substances. Until comparatively recently, however, the protective action of the ingredients of ice cream mix has not been recognized.

Reavens (1930) found that 4 to 20 per cent lactose increased the thermal resistance of E. coli. The probability that the ingredients of ice cream might afford microorganisms some protection against heat was recognized by the Committee on Dairy Products and Eggs (1930). The results of Oldenbusch, Frobisher, and Shrader (1930) gave only slight evidence of increased survival of various pathogens when heated in ice cream mix or in cream containing 30 per cent fat. Fabian and Coulter (1930 a) observed higher thermal death points for cultures of Escherichia coli and Aerobacter aerogenes when heated in ice cream mix than when heated in skim milk. Except for sucrose, these authors were unable to show any marked protective effect when the ingredients of ice cream were studied separately.

Sugar. Anzulovic (1932) reported that sugar, gelatin, serum solids, and fat showed some protective action for bacteria. Contrary to the results of subsequent investigators, Cochran and Perkins (1914) and Dougherty (1920) found that high concentrations of sugar offered no protection to yeast cells subjected to lethal temperatures. Weiss (1921 b) found that Bacillus botulinus was more resistant to heat in foods containing heavy syrups. Rahn (1928) stated that, "Sugar not only retards the action of heat upon microorganisms; it will take more heat to kill a bacterium or yeast in a sweetened fruit juice than in the same juice without sugar". Robertson (1927) heated Streptococcous thermophilus, Sarcina lutea, Escherichia coli, and Micrococcus aureus in increasing percentages of sugar and found that as the concentration of suger was increased the number of surviving bacteria also increased. Robertson stated that, "Hypertonic solutions as indicated by comparisons of heating cells in nutrient broth containing increasing concentrations of sucrose have a protective action up to and including 50 per cent sucrose. The protective action may be explained in part by the osmotic influence of the sucrose solution outside of the cell wall, and, in part, by the presence of capsules formed about the cells when grown in sucrose solution".

Neahkovitch (1927) showed that glucose tended to prevent the coagulation of cell colloids and aided in maintaining a

normal condition of stability of the cells of organized tissue.

Wallace and Tanner (1930) suspended several species of molds in 10, 25, and 50 per cent sugar, distilled water, syrups from fruit juices, and in salt water. Protective action was afforded by sugar for some molds and by salt water for others.

Rahn (1932 p.330) reported increased thermal resistance of yeasts and bacteria when heated in broth containing 50 per cent sucrose. The author suggested that although death may be due in part to dehydration, yet the cause of death is not the same as that of dry bacteria. Death results from some reaction with water, the rate of which has been retarded due to the diminished water content of the menstruum. Cook (1931) also explained his observations of increased resistance of yeasts in hypertonic glucose and sucrose solutions on a basis of dehydration. He stated, however, that there was probably some other factor involved beside osmotic pressure, since the killing times were not proportional to the osmotic pressures of the solutions employed.

Bailinsson (1929) observed that the addition of sufficient sucrose or glycerol to serum albumin or to egg white rendered these proteins stable to temperatures far above the usual coagulation points. Bancroft and Rutzler (1931) confirmed these observations and suggested an explanation based upon the peptizing action of sugar on albumin.

Süpfle and Dengler (1915) reported that mold spores were more resistant to heat in 5 per cent dextrose broth than in plain broth. These authors also observed a similar protective action of 5 per cent dextrose broth against the action of such disinfectants as phenol, "grotan", and "sagrotan".

Rosenblatt and Rosenblatt (1910) and later Toulouse (1929) found that high concentrations of sugar greatly retarded the destructive action of acids on cells. The latter author suggested that since the sucrose in a 36° Bé solution takes up 6 ± 2 molecules of water, this would account for nearly all the water present. The resulting competition between the sugar and the acid-water system for the available water would leave the germicidal acid without a vehicle for transportation into the cell. In this way the sugar afforded a protective action to the cell against a destructive agency.

Sumner (1911) and Loeb (1913) observed that cane sugar solutions protected Fundulus heteroclitus against the poisonous action of copper and zinc salts.

Milk. That milk might afford some protective action to organisms was suggested by Smith (1899). Later experiments by Russell and Hastings (1900, 1902) showed that organisms were protected by the pellicle which forms on milk heated in an open vessel. This was not wholly attributable to the slightly lower temperature in the pellicle, but partly to the decreased

water content in the immediate environment of the cell. Ficker (1906) reported that organisms causing cholera which had been washed from slants with milk and dried in milk were much more resistant than similar preparations in water, physiological saline, broth, urine, serum, or saliva. Wolff (1908) and later Corini (1915, 1921) concluded that milk afforded lactic acid bacteria an effective protective covering. Barthel and Stenström (1912) recognized that the organisms in milk naturally infected with Mycobacterium tuberculosis gave different results in thermal resistance studies than those in milk to which the organisms had been added from pure cultures. Holman (1914) stated that "albuminous fluids such as milk, beef serum, and ascites fluid, have a distinctly protective action for bacteria against heat". Ayers and Johnson (1914) suggested that the resistance of a few cells when heated in milk may be due to some protective influence of the milk. Brown and Peiser (1916) concluded that the lactic acid bacteria were rendered more resistant to heat by the protective action of the casein and fat in milk.

Proteins. Various proteins have been reported as protecting cells against heat or retarding the action of disinfectants. Ficker (1895) found that when the organisms responsible for cholera were heated in broth they survived approximately seven times as long as when heated in NaCl solution. Wright (1917) suggested that peptone and beef extract probably play an im-

portant role in regulating the chemical action between the cell and disinfectant when making a determination of the phenol coefficient. Dickson and coworkers (1919) concluded that spores of B. botulinus were more resistant to heat when mixed with animal and vegetable proteins. Winslow and Brooke (1927) found that the addition of a small amount of peptone or beef extract protected cells against the toxic action of distilled water. Dubos (1930) reported that commercial peptone contains substances which, in the oxidized form, are bacteriostatic for certain bacteria, but are not so in the reduced form. Sommer (1930) found that increasing the concentration of peptone in the medium increased the number of cells surviving a given heat treatment. Rideal (1930) stated that immersion in 1 per cent peptone stops the osmotic swelling of a cell. This may be due in part to strengthening of the cell wall, but it is more likely that the adsorption of capillary active materials from the peptone alters the permeability of the cell. Murray and Headlee (1931) were able to demonstrate a slight protective action of gelatin and peptone for Clostridium tetani spores subjected to heat. McCulloch (1932 a) made use of the protective action of egg white in his studies of the germicidal power of lye against Brucella abortus.

That blood serum delays the destruction of microorganisms was suggested by Smith (1908) as an explanation of the frequency with which non-sterile antitetanic serum was reported.

Browning and Gilmour (1913) and more recently McCulloch (1932 b) have reported that blood serum may decrease the bactericidal effectiveness of $HgCl_2$ as much as 100 times that observed in the absence of such a material.

Dead bacterial cells and even filtrates from young cultures increased the heat resistance of viable Bacterium coli and Micrococcus pyogenes according to Lange (1922). In most cases the addition of 1 cc. of a suspension of dead cells to a suspension of viable cells doubled the length of time required to sterilize at 58 to 60°C. Rahn (1932, p.338) stated that the same could be demonstrated for the action of disinfectants - the addition of dead cells decreased the toxic effect of a disinfectant solution.

Sodium chloride. Esty and Meyer (1922) heated Cl. botulinum in various concentrations of NaCl and found 0.5 to 1.0 per cent markedly increased the resistance. When 2 to 3 per cent were added the protective effect was lost, and more than 3 per cent decreased the resistance. Sherman and Albus (1924) reported that old cells were not sensitive to 5 per cent salt, whereas young cells were quite susceptible. This was attributed to "biologic rejuvenescence". Viljoen (1926) found that the addition of 1.5 to 2.5 per cent NaCl to pea liquor gave a maximum protective effect against heat. In many cases the increase in time necessary to kill spores protected by NaCl was quite marked. He observed that the resistance of all rather

than a few of the spores was increased. Peterson (1927) likewise reported protective action of NaCl in concentrations of 1.5 to 2.5 per cent. Murray and Headlee (1931) heated spores of Cl. tetani to 95, 100, and 105°C. in 0.85, 2.0, 3.0, and 4.0 per cent NaCl solutions. At the lower temperatures 2.0 per cent NaCl gave uniformly greater resistance. Ficker (1898) on the contrary found that when the organisms responsible for cholera were suspended in NaCl solutions they were more easily killed by heat than when suspended in broth. Shearer (1916-1917) reported that meningococci were extremely sensitive to 0.85 per cent NaCl at 37°C. Weiss (1921 a) concluded that NaCl greatly lowered the thermal resistance of the spores of Bacillus botulinus. Winslow and Brooks (1927) found that the addition of 0.145 to 1.45 per cent NaCl failed to revive organisms which had been injured by continued washing and centrifugation. Reichel (1909) observed the effect of salt on the distribution ratio of phenol between water/oil, and water/egg albumin phases. The addition of NaCl to suspensions of staphylococci rendered them more susceptible to the destructive action of phenol.

Other salts. Neuberg (1916) reported that high concentrations of sodium benzoate, sodium salycilate, sodium hippurate and potassium toluate prevented the coagulation of sheep serum by heat.

Glycerol and oil. The results of Bartlett and Kinne (1913),

showing the remarkable increase in thermal resistance of Staphylococcus aureus, Bacillus anthracis, and Bacillus subtilis when heated in glycerol, olive oil, cotton seed oil, and paraffin suspensions, emphasize the importance of water in the destruction of the cell. Spores which were killed in boiling water after 3 minutes were found alive after heating to 100°C. for 75 minutes in glycerol and for 30 minutes in oil. Bullock (1913) found that the autoclave was without special value in the sterilization of glycerol and other anhydrous fluids. Spores in such liquids subjected to steam under high pressure were not killed any more quickly than those in the controls which were held at the same temperature in the dry air oven. Almost identical results have been reported by Dreyer and Walker (1912-13). Reference has already been made to the work of Bellinsson (1909) who demonstrated the retarding effect of adding glycerol or sucrose on the coagulation of serum albumin. Siefert (1914) also has shown that some of the pathogenic bacteria can withstand higher temperatures in the presence of glycerol although the glycerol itself is quite toxic. Von Wunschein (1901) and Lode (1920) have called attention to the fact that glycerol lowers the toxicity of several disinfectants such as phenol, acids, alkalis, and various organic disinfectants mutually soluble in water and glycerol.

Source of organisms. von Esmarch (1889) and Gruber (1891) pointed out the fact that the ease with which organisms could

be killed was partly dependent upon the conditions under which they had grown. Weil (1899) confirmed other observations of von Esmarch (1889) which showed that the temperature of incubation influenced the ability of anthrax spores to resist heat.

Burke (1919) found that cultures grown in brain medium were more resistant to heat than cultures of the same strain in broth. Reiter (1920) stated that anthrax bacilli grown in "Weizenextraktagar" of Heider were more difficult to kill than cultures which had been cultivated on any of the ordinary media employed.

Esty (1920) concluded that the thermal death point of Clostridium Welchii was affected by the source from which it had been obtained. He reported that cultures grown in glucose liver broth for 24 hours had a thermal death point 1°C. higher than those grown in plain broth; also cultures isolated from milk were less resistant to heat than those isolated from human and bovine feces.

Seligman (1923) suggested that the pH of the medium in which the test organisms were grown was an important factor in testing disinfectant solutions. Lockeman and Pilcher (1923 a, b) emphasized the importance of considering the environment of bacteria in choosing a disinfectant. Protection to the organism may consist merely of chemical union of some ingredient with the disinfecting agent, thus removing the dis-

infectant from the sphere of action. In many cases protection may be afforded the cell by a change in the permeability of its membrane. Thus Noguchi (1905) explained the protective action of an excess of cobra venom on the hemolysis of blood cells. Although the blood cells were extremely sensitive to low dilutions of the venom, the addition of an excess not only prevented hemolysis but so completely altered the cell that its protective influence could not be removed by several washings.

Watkins and Winslow (1932) expressed the opinion that certain media contribute a protective substance which adheres to the cell and is carried over in transfers. This layer of protective substance may be washed from the cells.

By introducing graduated doses of metallic salts into media Ziegler and Dörle (1930) showed that the toxic action against cells was lost and that a phenomenon similar to drug habituation could be produced.

Theories of Cell Destruction

Several theories have been advanced to explain the processes involved in the destruction of the cells by heat or chemicals. Nernst (1890) originally suggested that the varying degrees of cell permeability to chemicals might be due to selective solubility in the various components of the cell mem-

brane. From this Overton (1900, 1901) reasoned that since most of the substances known to be toxic to the cell are soluble in fats, the cell membrane must be composed of a lipoid layer which regulates permeability.

Chick (1910) concluded that the destruction of cells by heat consisted of a reaction between the water and the protein and that the process was closely parallel to the coagulation of protein.

Rochaix (1912) divided disinfectant substances into electrolytes which acted by ionization and adsorption, and non-electrolytes which acted by adsorption only. In either case the protoplasm of the cell was coagulated.

Spaeth (1916) believed that death of the cell was always due to factors which caused an increased permeability. Mayer (1917) concluded that death from high temperature was due to an accumulation of acid (probably H_2CO_3) in the tissues. Brooks (1918) regarded the process of cell destruction as the result of a catenary series of reactions, the rates of which were governed by the slowest reaction in the series. Traube and Somogyi (1921) emphasized the importance of physical forces such as surface activity, adsorption, electrical potential, swelling, coagulation, and osmotic effects in the destruction of the cell. The results of Zoond (1927), confirming those of Spaeth, led to the conclusion that death of the cell was accompanied by an increase in permeability of the membrane which

allowed free diffusion of the cell contents.

In order to harmonize the comparative death rates of bacterial cells and higher forms of life, Rahn (1929) proposed a hypothetical scheme to illustrate the mechanism of death. He conceived of the cell as having one or more sensitive molecules which must be destroyed before the cell is killed. In general the more complex the form of life the larger the number of reacting molecules and the more the death curve will deviate from the monomolecular type.

Knaysi and Gordon (1930 b) concluded that the mechanism of disinfection was analogous to that of dyeing and tanning, in which it was shown that the agent was first adsorbed and then, if affinity existed between the adsorbed and adsorbent substances, chemical combination took place.

Rahn and Barnes (1932) stated that, "Death is defined in many different ways, and always by the loss of some property characteristic to the living organism. Compelled by the technique, bacteriologists usually define a cell as dead when it has lost permanently the power of reproduction,--other biologists usually consider a cell dead when it has lost the power of respiration (or fermentation) or the loss of plasmolysis or by adsorption of dyes". Rahn and Barnes studied the relationship of the various criteria of death by exposing yeast cells to heat, ultraviolet light, and chemical poisons. They observed that there was considerable difference in the time

required to bring about the various symptoms of death under uniform conditions, and that loss of reproductive power not only occurred earlier than any of the other symptoms but was not necessarily followed by an early loss of fermenting power, reducing power or selective permeability. Fulmer and Buchanan (1923) used as their criterion of death the stain adsorbing power of the yeast cell when suspended in a 0.01 per cent methylene blue solution. Buchanan and Fulmer (1930 Vol. II, p.334) stated that -- "it seems apparent that death is due to the gradual denaturation of cell proteins or to the destruction of cell enzymes".

Isaacs (1932) explained the whole process of disinfection on a basis of the destruction of the enzymes of the cell. He regarded the enzyme rather than the cell as the labile factor. Boutner (1933 p.21) stated that,-"A living organism is dead when the enzymes or the substances with which these react are exhausted, removed, disorganized; or when the chemical reactions are interfered with for other reasons".

Bancroft and Butzler (1931) and Bancroft and Richter (1931) presented a clear analysis of the factors involved in the denaturation of albumin and the partial or complete destruction of the cell. Their theory of the chemistry of cell destruction is based upon well established fundamental facts concerning the behavior of colloidal systems. However, as Miller (1931, p.89) pointed out,-"When one considers the high-

ly complex nature of protoplasm, its rapid adjustment to its environmental surroundings, its highly specialized nature for different individual tissues, and even for different parts of the same cell, it would seem most unlikely that any one theory could be proposed that would cover its behavior under all conditions toward the various substances that diffuse across it".

Several other theories have been advanced to explain the mechanism of disinfection, some of which are more specifically appropriate to points to be discussed subsequently.

The Physical Aspects of Cell Destruction

Adsorption. Beckhold (1909 a) assumed that adsorption played an important part in the disinfection process and stated that many of the phenomena of disinfection which had not been clear could be explained on a basis of colloid chemistry. He believed that chemical processes also played an important part in disinfection. If adsorption alone were the mechanism, then all bacteria would react in the same way to similar concentrations of the same disinfectant. In a contemporary paper (1909 b) he proved that such an assumption was untenable. Herzog and Betzel (1910) found that $AgNO_3$ was bound to yeast cells in a fashion which suggested an adsorption process. Cooper (1912) found that when a certain amount of phenol had been adsorbed on the cell the protoplasm precipitated. This

was accompanied by a great increase in the adsorptive capacity for phenol. Essentially the same observations were reported by Küster and Rotheub (1913) who concluded that a definite minimum amount of phenol must be adsorbed before the death of the cell occurs.

Süpfle and Müller (1920) proved that adsorption was responsible for the action of $HgCl_2$ by removing it from the cell with other adsorbents. They could revive anthrax spores which had been exposed to 2 per cent $HgCl_2$ for 40 days. They concluded that after long exposure $HgCl_2$ enters the cell but at first it is only adsorbed on the surface. Essentially the same observations were reported by Englehart (1922). Beckhold and Reiner (1922) likewise observed that surface active disinfectants could be retarded by the addition of surface inactive substances. They also observed that disinfectant action was decreased when the adsorptive surface was increased and the amount of disinfectant held constant. Hansen (1921) found that alteration of the surface tension of a cell by addition of alcohol increased the effectiveness of certain disinfectants. The importance of adsorption in the process of disinfection was stressed by Hall (1922) in the statement that, "One of the basic facts in disinfection by means of chemicals is that a certain distribution occurs between the organism and the disinfecting substance. The distribution may be either the result of adsorption or direct

chemical combination".

Gegenbauer (1922) demonstrated that in the case of formaldehyde the mechanism of disinfection was by direct combination with the cell constituents. When the time of action was less than 6 hours, the amount of aldehyde bound was directly proportional to the concentration employed.

Jezler (1926) showed that the addition of organic substances such as glucose diminished the adsorption of iodine by charcoal, whereas the addition of inorganic salts (NaCl, $CaCl_2$, or $MgCl_2$) appreciably raised it. Cooper and Mason (1928) studied the adsorption of various substances by colloidal solutions of proteins as a possible index to the nature of interaction between the cell and the chemical. They concluded that the germicidal or inhibitory power bears no relationship to its solubility in, or adsorption by, proteins in colloidal solutions. Wright and Hirschfelder (1930 b) were able to show that the adsorption of one substance affected the rate of subsequent adsorption of other substances, thus explaining the so-called "interference phenomenon".

Horst (1931) made a quantitative study of the adsorptive capacities of various types of cells. He found that for any one bacterial form the amount of iodine loosely adsorbed bore a constant ratio to the amount more firmly adsorbed. Gram positive cells adsorbed more of the iodine firmly than the Gram negative cells.

Stimulation. Many workers have observed that low concentrations of toxic substances exhibit a stimulating action on cell processes. The effect of certain non-electrolytes on nerve cells was studied by Mathews (1904 c), who found that 0.5 molar solutions tended to stimulate the cells, either directly by extraction of water, or indirectly by increasing the concentration of salts in the outer part of the nerve. Gainey (1912) found that the addition of small amounts of carbon disulphide, toluol, or chloroform exerted a stimulating effect on the total number of microorganisms present in soil.

Ether, in concentrations between 1.10 to 2.65 per cent, was found by M. M. Brooks (1918) to stimulate respiration of B. subtilis. The same author (1920, 1921) reported that 6 x 10-6 molar lanthanum nitrate increased the rate of respiration of B. subtilis; lower concentrations had no effect, and higher concentrations were toxic to the organism.

Observations under the ultramicroscope suggested to Nadson and Meissl (1926) that the first action of chloroform on yeast cells resulted in an excitation of the protoplasm.

Hotchkiss (1923), Winslow and Falk (1923) and Winslow and Haywood (1931) found that certain concentrations of various cations exerted at first a stimulating effect on bacterial viability. Further increase in concentration induced a decreasing stimulation which finally became toxic. The last named authors suggested that the explanation for this phenom-

anon probably rested on the theory of Bancroft and Richter (1931). According to Bancroft and Richter, when a narcotic enters the cell it is first adsorbed on the colloid to which it is most attracted. This causes a displacement of material already adsorbed by the colloid and, as a result, the effective concentration of the displaced material in the water phase is increased. This increase in concentration, according to the mass law, accelerates the chemical reactions which the substance is undergoing. For strong disinfectants this zone of stimulation, which obtains only in the incipient stages of adsorption, may be so narrow as to escape notice.

Coagulation. Whatever may be the mechanism of the death of the cell, it is quite universally agreed that the end result is the same. The colloidal suspension in the cell is irreversibly coagulated whether death has been induced by physical or chemical means. If the colloidal suspension is reversibly coagulated, the cell may have lost temporarily any or all of its vital manifestations, but will recover from dormancy when placed in a favorable environment. When cells have been subjected to very severe treatment with heat or chemicals there may be little doubt as to the irreversibility of coagulation. On the other hand, attempts to evaluate the resistance of cells to heat or chemicals usually involve the determination of minimal exposures. The irreversibility of the coagulation is not assured except possibly by failure of the

cell to grow on a single medium.

As shown by previous citations the incipient stages of the coagulation process frequently result in a stimulation of the cell. As agglomeration of the cell contents increases, the cell loses more and more of its functions, the coagulation becomes progressively less reversible and finally is completely irreversible.

Obviously then, death is not as sudden as the determinations of thermal death points and phenol coefficients might suggest. The fact that after treatment cells fail to grow on a given medium or on several media is incomplete evidence that they are dead. Determinations of minimal lethal exposures are especially subject to criticism because of the likelihood that the cell proteins may be only reversibly coagulated.

Herzog and Betzel (1912) were of the opinion that the first stages in the action of phenol were reversible and were followed by irreversible chemical combinations. Cooper (1912) and Cooper and Sanders (1927) likewise suggested that phenol was at first reversibly adsorbed by the cell, but that the death which followed was the result of de-emulsification of the colloidal suspension rather than chemical union.

In harmony with the work of Bancroft (1913), Lloyd (1915) suggested that the momentary shifting of the water between the continuous and discontinuous phases produced a constantly

changing condition of hydration and coagulation. This not only regulated the intake of the cell but governed the rate of activity or the extent of dormancy. When the coagulation became irreversible the cell was dead. Spaeth (1916) conceived of the important changes as taking place at the periphery of the cell. According to his hypothesis reversible coagulation of the colloids in the cell surface determines the degree of porosity of the membrane and hence regulates its permeability. He observed that substances which Osterhout (1912) found to decrease permeability ($MgCl_2$, $CaCl_2$, HCl, $La_2(NO_3)_6$), have distinct coagulating or dehydrating effects on colloids. Spaeth believed coagulation of the protoplasm resulted in death indirectly in that the increased fluidity of the cell constituents induced excessive permeability.

Freundlich and Rona (1917) and Meyerhof (1918) suggested that narcosis could be explained on a basis of the adsorption of sub-lethal amounts of a toxic substance and was the direct result of reversible coagulation of the cell colloids. Bechhold (1919, p.33) stated that,--"narcosis seems to me to be a typical example of simple solution, a process that is completely reversible".

In a study of the action of $HgCl_2$ on bacterial cells, Gegenbauer (1881) observed at first a "stunning effect" followed by a slow irreversible chemical change which led to

death. During the early stages growth was only inhibited and the cell could be revived by removing the chemical. After the irreversible chemical action had set in, however, attempts to revivify the cell were unsuccessful.

Ultramicroscopic observations by Hudson and Meisel (1926), Hirschfelder and Bechord (1927), Hirschfelder and Wright (1930 a), and Bancroft and Richter (1931) indicate an unmistakable tendency of the cell contents and of non-living colloidal suspensions to coagulate at first reversibly and later irreversibly when exposed to mercuric chloride, chloroform, phenol, triphenylmethane, merourochrome, acriflavine, and heat.

Lepeschkin (1926) outlined a theory of disinfection based upon the conception of reversible and irreversible coagulation of the protoplasm. More recently, however, Bancroft and Richter (1931) have summarized the available knowledge of disinfection processes and have formulated a theory of cell destruction based upon colloidal behavior. According to these authors stimulation, antisepsis, and disinfection are different manifestations of varying degrees of coagulation of the colloids. Factors which induce peptization of the colloid may counteract coagulating agencies and thereby afford a protective action. The protective action of dextrose demonstrated by Bancroft and Rutzler (1931) was explained on this basis.

In determining the effectiveness of a disinfectant it is important to remember that the coagulation may not have been carried to the irreversible stage. Failure to grow on a given medium merely indicates the inability of the medium to peptize the coagulated colloids. Another medium may induce the reversible change and growth will ensue.

This conception of the varying degrees of reversibility of the colloids of the cell elucidates the observations of earlier investigators. Hewlett and Hall (1911) found agar far superior to broth in testing the viability of anthrax spores after treatment with disinfectant. Süpfle (1914, 1916) emphasized the importance of the culture medium employed in determining the resistence of various organisms. Fay (1927) and Fabricius and Hemmer (1931) reported that the delayed germination in high dilution plates of ice cream could be avoided by using 1 per cent sucrose agar. Morrison and Rettger (1930 a) found broth inferior to milk as a medium for heat resistance studies with one of the organisms employed. They attributed the extreme variability in spore resistance after heating to the lack of specific favorable conditions in the culture medium rather than to inherent variability of the spores to withstand heat.

Delayed germination may be due to a slowly reversible coagulation or to the inability of the medium to induce peptization readily. Burke (1919) found that heating spores of

B. botulinus to sub-lethal temperatures greatly delayed germination. Esty and Meyer (1922) reported that sub-cultures of heated spore suspensions germinated as late as 378 days; Dickson and associates (1928) reported germination after 330 days. Magoon (1926) found that spores of Bacillus mycoides subjected to moderate temperatures acquired a greater resistance to heat. Isaacs (1930) observed an increased tendency for delayed colony formation in the cultures which had been heated to higher temperatures. Morrison and Rettger (1930 b) concluded that dormancy of bacterial spores is largely, if not entirely, determined by the conditions in the environment. The work of Liesegang (1930) presented a very striking illustration of the probable dissemination of pathogenic organisms ineffectively destroyed. Fowl cholera organisms exposed to 0.1 per cent $HgCl_2$ for 7 minutes were no longer viable on ordinary media, but when injected into the bird, growth was evident within 3 hours. In this case the reversibly coagulated cell colloids were peptized by the tissues but not by the artificial culture medium.

Imbibition of water. The importance of the water content of the cell as a factor in heat resistance was recognized by Adametz (1889), Lewith (1890), Cramer (1891), and by Davenport and Castle (1895). Spores and vegetative cells low in water content consistently were found to withstand higher

temperatures or longer exposures than similar cells containing more water. Reichel (1909) attributed the action of phenol to the fact that the coagulated albumin was incapable of imbibing sufficient water.

True (1914) submerged the roots of Lupinus albus in distilled water and observed that the semipermeability of the protoplasmic membrane was greatly impaired. He attributed the harmful action of distilled water to the solution from the membrane of materials requisite for efficient selective permeability.

Eisenberg (1918) reasoned that the colloids of the bacterial protoplasm existed in a degree of swelling which was optimum for its most efficient functioning. Imbibition of more water or the loss of water already present altered the degree of dispersion and resulted in a disturbance of normal metabolic function. He further observed that the specificity of action of certain disinfectants to Gram positive and Gram negative cells might be due to the sensitivity of the former to swelling agents and of the latter to precipitating agents. The variation in the tendency of Gram positive and Gram negative cells to imbibe water was further associated with the higher lipoid content of the former. Breinl (1920) found that the adsorptive capacity for halogens was significantly greater for Gram negative than for Gram positive cells.

Fulmer, Nelson, and Sherwood (1921) observed that the

optimum concentration of NH_4Cl for the growth of yeast was also the concentration which induced the least swelling of gluten held under similar conditions. Shrinking of the protoplasm of cells treated with chloroform was the first physical change noted by Nadson and Meissl (1926) in their observations under the ultramicroscope. Rideal (1930) stated that the osmotic swelling of cells could be stopped by immersion in 1 per cent peptone solution. This action was attributed to alterations at the cell surface resulting from the adsorption of the peptone which, in turn, effected a change in the selective permeability of the cell membrane. In studying the mechanism of denaturation of egg albumin, Loughlin (1932) concluded that there was a progressive imbibition of water until the volume was approximately doubled.

As Bancroft and Richter (1931) have suggested, the destructive action of distilled water is due to the coagulation of the colloids of the cell. Experimental evidence based on the narcotic effect of distilled water on nerve cells indicates that the coagulation is only reversible, but may become irreversible after prolonged action. Assuming that the electrolytes of the cell are responsible for the stabilizing charge of the colloidal particles, Bancroft and Richter suggested that there must be a minimum concentration below which the adsorption of ions is not sufficient to peptize

the colloids to the proper degree for continued stability. When the addition of distilled water dilutes the ions to this value the colloids flocculate.

Przylecki (1931) stated that the results with various ions can be readily explained if it be assumed that the state of dispersion of proteins is a function of their affinity for water and of the free charge on the particles. The reduction of the free charge below a certain critical minimum resulted in conversion of the protein sols into gels.

It is obvious that the extent of imbibition of water is an important item in regulating the degree of dispersion of the cell colloids. If the degree of dispersion is a criterion of stability, it is not surprising to find that many cells are extremely sensitive to distilled water.

As early as 1888 Frank reported that distilled water was toxic to lupine seedlings. Although this point was criticized by Schulze (1891), it was confirmed by Hibbard (1915) who observed that the toxic action of distilled water could be avoided by changing the water at frequent intervals. He assumed that the cells excreted a toxic substance when suspended in distilled water and that its removal was effected by frequent exchange of water. Daniels (1908-1909) explained the sensitivity of Paramecia to distilled water on the basis of a sudden change of environment.

Among the early investigators to note the sensitivity of bacterial cells to distilled water were Fisher (1898, 1908), Leuch (1905), Shearer (1916, 1917), Winslow and Cohen (1918), Zeug (1920) and Weiss (1921 a). Falgen (1925) found that the death of cells was much more rapid in water suspensions than in Ringer's solution. Winslow and Falk (1923 a) observed that when the reaction of water was buffered at pH 6.0, the rate of death of cells was very slow, but at pH values more or less than 6.0 the cells died at increasing rates. Winslow and Falk (1923), Shaughnessy and Falk (1924), and Shaughnessy and Winslow (1927) found that the organisms less sensitive to distilled water were capable of adjusting the reaction in the immediate environment of the cell to one more nearly optimum for self preservation. Bacillus cereus which was found to be extremely sensitive to distilled water had a much more permeable cell wall and was less capable of creating such a protective zone for itself. Winslow and Falk (1918) suggested the ingress of water as one of the possible explanations of the death of cells transferred from a 0.1 isotonic $CaCl_2$ to a 0.001 isotonic $CaCl_2$ solution.

Mudge and Lawler (1928) explained the apparent diminution of numbers of cells in dilution blanks to some factor responsible for the clumping of the cells, with the result that fewer colonies were formed when transplanted in agar plates. In a study of the selection of water for dilution

blanks Butterfield (1932) found relatively few sources of tap water satisfactory for this purpose. He emphasized the importance of testing the reaction of water in dilution blanks after sterilization and stated that the pH should not exceed 7.5. Many cells die in dilution blanks if the reaction is between pH 8.2 and 9.0, and when it exceeds pH 9.0 the death rate is very high.

The existence of a protective zone around certain bacterial cells which inhibits the toxic action of distilled water was further suggested by the results of Winslow and Brooke (1927) and Watkins and Winslow (1932). They found that washing of cells by three or more centrifugations rendered them much more susceptible to the toxic action of distilled water. Schumacher (1928) observed that repeated washing of yeast cells removed the endoplasm which contained the lipoid-protein complex of the cell surface. Obviously, such an alteration of the cell surface would materially affect its selective permeability. Ballantyne (1930) washed Bacillus typhosus in 0.85 per cent NaCl and in distilled water. He found that such treatment materially shortened the survival period when resuspended in these fluids. As a result of his studies he also suggested that great dilution of cells appeared to be almost equivalent to washing in its effect.

Osmosis. If the transfer of water across the cell membrane exhibits such a profound effect on the stability of its

colloidal suspension, it would seem logical to expect osmotic forces to play an important role in the resistance of the cell to its environment. A summary view of the available literature as given by Falk (1925) at once suggests that bacterial cells as well as many other forms of microorganic life are characterized by a rather remarkable resistance to osmotic forces. Obviously, there are many exceptions to such a generalization, but in most instances the exceptions are found among forms whose restricted habitat obviates the necessity for such protection. The ubiquitous nature of bacteria, yeasts, and molds necessitates a wide range of tolerance for various environmental factors. The individual cells of higher plants and animals on the other hand need no such protection. Loeb and Wastenays (1912) and Loeb (1913) observed that muscle tissues absorbed or lost water in solutions hypotonic or hypertonic with the cell, whereas there was neither a gain nor loss in weight in isotonic solutions. Stiles and Jorgensen (1917) showed that equimolecular solutions of different substances did not induce the same loss of water by plant cells. They were able to show that the more complex the molecule in homologous series the greater the loss of water induced by equimolal concentrations.

Beck (1921) found that the Og value for cells varied in different parts of the same plant. He stated (p. 317), - "The Og seems to depend upon the nature of the plant as well

as upon the environment in which the plant grows". According to Seliber and Katznelson (1927) the osmotic value of yeasts may be altered by growing in media of various compositions. The addition of glucose to a medium gave a lower osmotic value than the addition of equimolar concentrations of sucrose. The work of Lochhead and McMaster (1931) in which they found 79 per cent of 191 samples of honey to contain 50 to 10,000 yeasts per gram illustrates the rather wide distribution of yeasts capable of resisting high osmotic pressures.

The literature contains many reports of bacteria which are likewise capable of resisting high osmotic pressures. Mention is made here of only the description by James (1930) of a member of the colon-aerogenes group capable of growing in a 35° Brix sugar solution. That the resistance of certain bacterial cells to osmotic pressure might be attributed to surface phenomena was suggested by Brundy (1908), Eisenberg (1910), and Gutstein (1927) in the claim that Gram positive cells are not plasmolyzable. These observations, however, are not in agreement with those of Knaysi (1930 a).

The very exhaustive study of the toxicities of various ions by Eisenberg (1918) suggests that although a purely molecular (osmotic) force exerts a noticeable influence on the transportation of water into and out of the cell, it is greatly overshadowed by the changes induced at the cell surface by

the anions and cations. Eijkman (1916) on the other hand concluded that the ability of various salts to inhibit growth of bacterial spores varied directly as their osmotic pressures. Traube and Somogyi (1921) likewise concluded that osmotic pressure is a decisive factor in the activity of those disinfectants which do not depend upon purely chemical factors for their bactericidal property.

In discussing the apparent resistance of certain cells to osmotic pressure, Höber (1924, p.406) suggested that the explanation lies in the failure to meet the first requirement of plasmolysis, which is semipermeability. The importance of ions in regulating the selective permeability of the cell is emphasized by the results of Seliber and Katznelson (1927). They not only observed that equimolar solutions of dextrose and sucrose gave different 0_g values for suspended yeast cells, but that the addition of NaCl increased the Og value in each case. Höfler (1926) found monosaccharides more permeable than cane sugar. A more detailed study of this point was made by Lucké and McCutcheon (1932). They submerged cells in hypotonic dextrose solutions so that practically all of the osmotic pressure was caused by the dextrose, then added varying amounts of univalent and divalent ions. In this way they were able to demonstrate that univalent chloride salts increased the permeability of cells to water, whereas divalent chlorides reduced the permeability. Such a concept clarifies

the observations of many previous workers to the effect that cells may exhibit a variable resistance to osmotic pressure. If the semipermeability of the cell membrane is determined by ion effects it is not surprising to find that equimolal concentrations of various substances do not exhibit the same osmotic action on cells.

Permeability

Theories. Physical changes which take place at the surface of the cell or at the interface between the cell and its menstruum obviously might affect the permeability of the cell and hence the exchange of water or nutrients. Several theoretical concepts have been proffered which at least assist in a visualization of the fundamental processes that ultimately exert such a profound influence on the resistance of the cell.

Overton, (1895, 1899, 1901) advanced the theory that the cell membrane consisted of a lipoid complex which governed the transfer into and out of the cell. Working with Spirogyra and later with animal cells his observations led to the conclusion that fat soluble substances only were capable of entering the cell. The failure of plasmolyzed cells to regain their normal shape after continued standing in the plasmolyzing solutions was taken as evidence that the solute did

not enter the cell. Gösel (1913) attributed the action of certain organic disinfectants to their ready adsorption by lipoids.

Overton's lipoid theory of permeability has met with criticism from many sources because the limitations which it placed on penetration were not in harmony with observed facts. Osterhout (1911, 1912, 1913, 1914, 1915 a,b,c; 1917) studied the effect of various ions on permeability and came to the conclusion that plasmolysis was determined to a large extent by the nature of the ion effects on the cell wall as well as by the concentration or molecular osmotic pressure. He found that neither a 0.196-molal $CaCl_2$ nor a 0.375-molal NaCl solution would plasmolyze Spirogyra, but if the osmotic pressure of the NaCl solution were reduced by adding 10 per cent by volume of the $CaCl_2$ solution plasmolysis readily occurred. Osterhout regarded the lipoid theory as untenable since all of the salts studied were shown to penetrate and none was fat soluble. Lepeschkin (1911) criticized the view that the plasma membrane was a simple mosaic structure of lipoids and proteins. He believed it was a complex colloidal structure and that its permeability was dependent on forces other than mere solubility.

The early work of Loeb (1902) suggested that the penetration of the cell might be associated with the degree of dispersion of the colloids in the surface films.

The hypothesis of permeability outlined by Lloyd (1915) and later described by Free (1918) conceives of the protoplasm as consisting of two or more phases. These phases are intimate mixtures of two or more colloids which form liquid globules in a liquid medium, probably water. The relative volumes of these various phases depend upon the distribution of water between them. If the water passes from one phase to the other, there will be a corresponding change in the relative volumes of the phases. This in turn affects the space between the globules. If, as a result of continued swelling the particles of the discontinuous phase become tightly packed, the interspaces will be small and the permeability difficult. On the contrary, shrinking of the globules would increase the size of the interspaces and the permeability of the membrane would be increased. If protoplasm is analogous to simpler colloids it is reasonable to expect that changes in the concentration of electrolytes would induce such alterations in the distribution of water. According to this theory penetration of a substance would depend upon its solubility in the continuous phase of the colloidal complex. Saturation of the continuous phase would result in the withdrawal of more water from the globules, thereby widening the channels for the entry of still more of the penetrable substance. Water, glycerol, urea, and certain alcohols doubtless enter because they are soluble in both phases. Sodium chloride in-

creases permeability because it withdraws water from the globules. Calcium chloride, on the contrary, causes globules to swell and hence closes the interspaces.

The theory of permeability outlined by Spaeth (1916) differs from that of Lloyd and Free primarily in the matter of the mechanism. Spaeth considers the permeability of the cell as dependent upon the fluidity of the cell contents which, in turn, is dependent upon the degree of dispersion of the colloidal complex. Anything which affects the permeability of the cell such as heat, ions, or disinfectants, does so by inducing either aggregation or peptization of the colloidal particles. If the colloidal dispersion is increased there will be a corresponding increase in the fluidity. Since the mobility of ions is affected by viscosity it is clear that this would favor diffusion in either direction. Similarly, permeability may be increased by agencies which cause extreme coagulation of the colloids. In such a case the particles of the dispersed phase are so completely agglomerated that they settle out, the fluidity of the continuous phase is thereby increased and the cell is injured. Spaeth concluded that increased permeability was always associated with injury to the cell.

By conductivity methods Osterhout (1918) and Shearer (1920) studied the effects of various ions on permeability. All monovalent ions except H produced a rapid increase in con-

ductivity which was at first reversible and later irreversible. Bivalent cations and the H-ion at first slightly decreased the conductivity then produced an irreversible increase in conductivity.

Clowes (1916 a, b, 1916), on the other hand, regarded the permeability of the cell as being regulated by the inversion of phases. He concluded from his experiments with artificial membranes that, -"the variations in the permeability of the protoplasmic membrane are attributable to the action of electrolytes and metallic products on delicately balanced interfacial soap films and emulsion systems, and that proteins play no part in the valve-like mechanism controlling permeability other than to afford a supporting filamentous or mesh-like structure". According to Clowes, in case of a polyvalent salt the cation is adsorbed and results in a change of phase such that oil becomes the continuous phase and water the discontinuous phase. For monovalent salts the anion is more strongly adsorbed and water becomes the continuous phase. Free (1916) objected to this theory on the basis that it necessitates a more sudden change in permeability than experience would justify. A cell necessarily would cease at once to be permeable to a substance soluble in its continuous phase, and immediately become permeable to another substance soluble in the newly created continuous phase. According to Free such sudden changes are not

in harmony with common observations.

Beehhold (1919, p.40) followed in general the concept outlined by Clowes and regarded the periphery of the cell as an emulsion or gel-like system consisting of two continuous phases, in which fluctuations in the permeability of water and its solutes may be caused by variations in the metabolic products. His ideas on ion antagonism and the effect of these ions on the change of phase in the cell membrane coincided with those outlined by Clowes.

Cations were observed by Kahn (1921) to lower the permeability of the cell by inducing coagulation of the plasma membrane. Anions, on the other hand, increased the permeability by increasing the degree of dispersion of the colloids. This work was later confirmed by Mac Dougal (1923).

According to Winslow and Dolloff (1928) the bivalent ion induces the same effect on the cell as the monovalent ion but is eight to ten times more powerful, whereas the H and Hg-ions are 1000 times more powerful. All cations stimulate growth in low concentrations due to an increased permeability, whereas the toxic action of higher concentrations is due to decreased permeability. The decreased permeability is followed by an increased permeability which may be the result of a rupture of the cell or some irreversible physical change in the cell membrane.

Summary of the Literature

From the bits of evidence garnered by various investigators it is possible to weave an harmonious picture of the mechanism of cell destruction as well as the process by which protective agencies inhibit or retard death of the cell. Although the point of major interest in this investigation is the protective action afforded bacteria by the sugar in ice cream, a composite view of the whole picture is prerequisite to a clear understanding.

Experimental evidence seems to have established the following points:

Cells contain colloidal suspensions and are surrounded by membranes of variable composition and organization.

Destructive agencies either unite chemically with cell constituents, are adsorbed at the surface, or induce a change in the degree of dispersion of the colloidal complex. Stimulation, narcosis, and death are manifestations of the various degrees of coagulation induced.

Coagulation is reversible at first and finally at the death of the cell becomes irreversible. The failure of a cell to grow on a given medium does not es-

tablish the fact that it has been irreversibly coagulated. Since the peptizing qualities of different media vary, it is difficult to determine the minimal exposure requisite to kill a cell.

Surface phenomena regulate the permeability of the cell. Ions play an important role (1) by inducing changes in the character of the surface layer, (2) by altering the partition coefficient of a disinfectant, (3) by affecting the stabilizing charge of the colloidal complex, and (4) by influencing the degree of hydration.

Water is not only essential as a vehicle for cell nutrients but is intimately associated with the stabilizing charge on the cell colloids. This in turn regulates the degree of dispersion and hence the stability of the system. Certain bacterial cells are extremely sensitive to suspension in distilled water especially in high dilution.

Many disinfectants bring about their action by being adsorbed on the surface of the cell. The adsorption is at first reversible and the disinfectant may be removed by washing or by selective adsorption. Prolonged action may lead to an irreversible change and the death of the cell.

Protective agencies bring about their action (1)

by inducing peptization, (2) by altering the permeability, (3) by removing the disinfectant (4) by chemical union or adsorption, changing the rate of adsorption of the partition coefficient, and (5) by lowering the chemical potential of the destructive agent.

METHODS

In this study of the protective action which sugar affords microorganisms against destruction by heat, methods have been devised to evaluate the increased thermal resistance under various experimental conditions. Although each experiment was designed to demonstrate the effect of some variable under properly controlled conditions, there was a certain degree of uniformity in the experimental procedure. The following descriptions of the methods and materials used apply more or less generally to many of the experiments. The special procedures which individual experiments necessitated will be described at the appropriate time.

Bacteriological Methods

Media. In all of the plating procedures beef extract agar of the following composition was employed.

Market agar, 1.5 per cent,
Bacto peptone, 0.5 per cent,
Bacto beef extract, 0.3 per cent,
Water to volume.

In those instances in which comparisons were made between the counts on plain and carbohydrate media, a large batch of the plain agar was divided into equal parts and, in the case of

the carbohydrate media, 1 per cent of the desired carbohydrate added to each portion. The reaction of all media was adjusted to pH 7.0 before filtration. All media were sterilized in the autoclave for 20 to 30 minutes at 15 pounds pressure.

Plating technique. Except as otherwise noted, distilled water was used in making dilution blanks. Occasionally the final reaction of these blanks after sterilization was tested colorimetrically and found to be about pH 6.0. An automatic pipette calibrated so as to yield dilution blanks containing 99 cc. of water after sterilization was used in filling all dilution bottles. The use of 9 cc. blanks was eliminated by employing 1.1 cc. pipettes in making dilutions.

Special attention is called to the fact that in most cases only single plates of each medium employed were poured from appropriate dilutions. This should be borne in mind in interpreting the results. As will be pointed out later, cells suspended in hypertonic solutions are frequently injured when transferred to dilution blanks. This necessitated speed in making dilutions and pouring the agar into the plates. One of the difficulties involved in pursuing this study was to reduce the time required for plating of an individual sample to a minimum and yet keep it standard for the comparative determinations. This was accomplished as far as possible by making the dilutions as rapidly as was commensurate with care and accuracy and then pouring the agar im-

mediately. In nearly all of the experiments, interval time clocks were used to standardize the time of plating and also to regulate the period of exposure of the samples to heat.

All plates were incubated 48 hours at 37°C. In several of the experiments where delayed germination was suspected, counts were made again after an additional 3 day incubation at room temperature. Bausch and Lomb, Spencer, and Leitz, low-power, binocular stereo-magnifiers were used in counting the colonies on the plates. By standardizing the area of the fields provided by the various combinations of oculars and objectives, it was possible to determine the factors for the proportional part of the area of the entire plate observed in a single field. These factors were found to be especially advantageous in enumerating the colonies on heavily seeded plates.

Cultures employed. The two cultures used in most of the experiments were designated as E. coli 52 and E. coli 57. These are representative of the communis and communior varieties respectively of the Escherichia genus, and were obtained from the regular stock cultures carried in the Bacteriology Department of the Kansas Agricultural Experiment Station. The other cultures employed were obtained from the same collection.

Preparation of Hypertonic Solutions

Concentrations expressed in per cent. In several experiments reference is made to the use of a 100 per cent sugar solution. This refers to a weight-volume ratio and the solution was prepared in the following manner: Approximately 250 cc. of water were added to 1 kilogram of the desired sugar; this was boiled for a few minutes until a clear solution was obtained. The resulting syrup (approximately 900 cc.) was diluted to 1000 cc. total volume. Such a solution therefore contained 1000 grams of sugar in a total volume of 1000 cc., and each cubic centimeter represented 1 gram of sugar. Although not in conformity with the usual concept of per cent concentration, this preparation was regarded as a 100 per cent solution. All of the other sugar solutions employed, the concentrations of which are expressed in percentages, were prepared by suitable dilution of this 100 per cent sugar solution. Thus a 50 per cent sucrose solution refers to a dilution of equal parts of water and 100 per cent sucrose solution prepared as previously described.

Molal solutions. The sugar solutions, the concentrations of which are expressed in molality, were prepared by dissolving the indicated number of gram-molecular weights in 1000 cc. of water.

Heating Conditions

Temperature regulation. In the experiments conducted at the Kansas Agricultural Experiment Station an electric, thermostatically controlled, De Khotinsky oil bath was used for heating the samples. In the experiments conducted at the Iowa Agricultural Experiment Station, all tubes subjected to heat were submerged in a 10 gallon tank of water without thermostatic control. Since the time of exposure in all cases was comparatively short it was not difficult to maintain the temperature within the limits of ± 0.2°C.

Heat penetration. In order to reduce the factor of heat penetration to a practical minimum, small samples were used in all heating trials. In some cases, 1.5 to 2.0 cc. samples were placed in small hermetically sealed tubes and completely submerged in the water or oil bath for the desired heating period. In other cases, thin-walled test-tubes were submerged in the oil bath to within 1 inch of the top of the tube. The tube was approximately half filled when a 2.0 cc. sample was employed, so that with the large area exposed and the thin column of liquid very little time was required for penetration of the heat. For many of the experiments special tubes were prepared by blowing a bulb about 1.5 inches in diameter on the end of a soft glass test-tube. The small

sample in the relatively large bulb of very thin glass acquired the temperature of the water bath very quickly. In some cases 0.1 cc. or 1.0 cc. samples were heated in these blown tubes and 9.9 or 9.0 cc. of sterile water were added directly to the tube after heating to give a 1-100 or 1-10 dilution respectively.

RESULTS

Hypertonic Solutions in Ice Cream Mix

Effect of plain and carbohydrate agar on apparent survival. A sample of commercial ice cream mix was collected before the sugar had been added, and incubated at room temperature until the bacterial count reached 14,000,000 per cc. This sample was divided into four parts and equal volumes of sterile sucrose solution were added to give 15, 25, and 50 per cent concentrations of sugar. (See methods). An equal volume of water was added to one portion which was designated as 0 per cent.

One-tenth cc. portions of each were heated to 54°C. for 9 minutes in the thin walled blown tubes submerged in a water bath to within 1 cm. of the top of the tubes. After cooling for 1 minute in iced water, 9.9 cc. quantities of sterile water were added to each tube, thereby giving 1-100 dilutions. From these, higher dilutions were prepared and plain agar plates poured.

The results in table 1 show the counts before and after heating, and the survival per million calculated on a basis of the count before heating. In the sample containing no

added sugar the survival per million was only 450, whereas the addition of 15, 25, and 50 per cent of sugar resulted in a material increase in the survival of cells.

Table 1. Effect of sugar on heat resistance of bacteria in ice cream mix.

Per cent sugar added to mix	Count per cc.		Survival per million
	Before heating	After heating, 54°C., 9 minutes	
0	14,000,000	6,300	450
15	14,000,000	490,000	35,000
25	14,000,000	620,000	44,000
50	14,000,000	500,000	36,000

Although the results suggest that the sugar added to the samples afforded the organisms some protection against the destructive action of the heat, it is conceivable that enough sugar might have been carried over from the sample in the low dilution plates to slightly improve the medium. This point was further suggested by the marked inconsistencies observed in the plates from high dilutions. The 1-1000 dilution plates for the samples to which 15, 25, and 50 per cent sugar had been added showed 487; 617; and 503 colonies re-

spectively. One would therefore expect to find approximately 48; 60; and 50 colonies on the corresponding 1-10,000 plates in each series. There were, however, only 3; 2; and 1 colonies respectively on these 1-10,000 plates. Such inconsistencies in counts on various dilutions were very frequently encountered in many experiments in which cells suspended in hypertonic solutions were diluted more than 1-1000. The experiment as outlined was repeated except that plain agar and 1 per cent sucrose agar plates were poured from each dilution.

The results in table 8 show that if carbohydrate agar is used, more of the organisms are able to recover from the destructive effects of the heat. However, it is significant that a materially increased survival was found in the ice cream mix containing 50 per cent sugar. This cannot be accounted for on a basis of the sugar carried over from the sample. As the following computation will show, there is much less sugar carried over into the plain agar from this sample than the 1 per cent of sugar in the sucrose agar medium. The calculated amount of sugar carried over from the sample containing 50 per cent sucrose into the 1-10,000 plain agar plate is 0.00005 gram, whereas each of the carbohydrate agar plates contained approximately 0.1 gram or 2000 times as much sucrose. Had sugar in the medium been the only factor involved, one would expect larger numbers, approximating 40,000 per

Table 2. Comparison of results with plain and carbohydrate agar on the recovery from injury when cells are heated (54.5°C., 9 minutes) in the presence and absence of sugar.

Per cent sugar added to ice cream mix	Count per cc.					
	Plain agar			1 per cent sucrose agar		
	Before heating	After heating, 54.5°C., 9 minutes	Survival per million	Before heating	After heating, 54.5°C., 9 minutes	Survival per million
0	110,000,000	24,000	220	110,000,000	600,000	5,500
15	110,000,000	590,000	5,500	110,000,000	880,000	8,000
25	110,000,000	650,000	5,900	110,000,000	870,000	8,000
50	110,000,000	4,800,000	44,000	110,000,000	4,300,000	39,000

million, to have survived in each case in which sucrose agar was used for plating. Although this was obviously not the case, it is quite evident that the presence of an adequate supply of sugar in the medium greatly stimulated the recovery of injured cells.

Another sample of ice cream mix was incubated until the count was approximately 170,000,000 per cc. Sugar solutions were added to aliquot portions of this ice cream mix as previously described, and the samples heated to 54.5°C. for 9 minutes. These were plated with plain, dextrose, sucrose, and lactose agars. The results likewise indicate that the presence of sugar in the medium greatly increased the number of colonies on the plates, and further, that the ice cream mix containing 50 per cent sucrose exhibited marked protective action for cells.

The results in table 3 are especially significant when interpreted in the light of the theory of cell destruction outlined by Bancroft and Richter (1931). The microorganisms in the ice cream mixes containing 0, 15, and 25 per cent of sugar were definitely injured by the heat exposure. The injury apparently consisted of a degree of coagulation which was reversible under certain conditions. Plain agar evidently was able to peptize 55,000, 250,000, and 470,000 of the injured cells, whereas carbohydrate agar induced peptization of from

Table 5. Comparison of results with plain and various carbohydrate media on the recovery of organisms heated (54.5°C., 9 minutes) in the presence and absence of sugar.

	Per cent sugar added to ice cream mix	Count per cc. on			
		Plain agar	1 per cent dextrose agar	1 per cent sucrose agar	1 per cent lactose agar
Before heating	0	170,000,000	180,000,000	180,000,000	170,000,000
After heating	0	55,000	3,500,000	3,100,000	3,200,000
After heating	15	250,000	2,600,000	2,300,000	3,100,000
After heating	25	470,000	2,800,000	2,900,000	3,700,000
After heating	50	4,300,000	11,000,000	10,000,000	10,000,000

2,800,000 to 3,700,000 cells per cc. The rather marked increase in the number of cells in the mix containing 50 per cent sucrose cannot be accounted for entirely on a basis of the type of medium employed. It seems quite evident that the degree of coagulation of the cells in this case must have been more readily reversible.

That the amount of sugar carried over into the agar plate from the sample is not the sole limiting factor in the recovery of the injured organisms is illustrated by a comparison of the results obtained on plain agar with the mixes containing 25 and 50 per cent sugar. A 1-1000 dilution plate was used to count the 470,000 cells in the sample containing 25 per cent sucrose, whereas a 1-10,000 dilution plate was used to determine the 4,500,000 count on the sample containing 50 per cent of sucrose. Calculating the percentage of sugar in the agar in these two plates which was traceable to the sample (assuming 10 cc. of agar in each plate), the former contained 0.0025 and the latter 0.0005 per cent sucrose. That is, there was five times as much sugar carried over in plate containing approximately one-tenth as many colonies.

The concept outlined by Bancroft and Richter presents a logical basis of explanation of the data in tables 1, 2, and 3. The number of colonies which grow after cells have been exposed to mildly destructive forces is the resultant of two

opposing forces, coagulation and peptization. The more severe the exposure, the greater the degree of coagulation and hence the more difficult the peptization. If this explanation is sound one would expect; (1) that in the presence of substances which tend to retard coagulation, peptization of injured cells would be augmented; (2) the addition of certain substances to a medium would favor peptization and hence induce the growth of larger numbers of injured cells.

Effect of adding sucrose directly to the agar plates.

From the preceding experiments it was evident that the presence of sugar in the agar tended to aid in the peptization of injured cells. A series of ice cream mixes containing 0, 15, 25, and 50 per cent sugar were prepared from the same stock mix as previously described. One-tenth cc. samples in the thin walled, blown tubes were exposed in a water bath to 55°C. for 9 minutes. To one of the duplicate plates prepared from each dilution was added 0.5 cc. of a 50 per cent sucrose solution. Plain agar was poured into each plate.

The results shown in table 4 indicate that the presence of sugar in the agar enables the recovery (peptization) of cells which have been reversibly coagulated by heat. Duplicate plates of the same medium without sugar showed markedly fewer colonies in all cases except for the sample containing 50 per cent sugar. Apparently the presence of this amount (50 per cent) of sugar sufficiently protects the cells

Table 4. Effect of adding sucrose directly to agar plates on the growth of organisms: ice cream mix heated (55°C., 9 minutes) with and without sugar.

Per cent sugar added to ice cream mix	Count per cc. on					
	Plain agar			Plain agar plus 0.5 cc. of 20 per cent sugar solution added to each plate		
	Before heating	After heating	Survival per million	Before heating	After heating	Survival per million
0	66,000,000	17,000	260	60,000,000	1,000,000	17,000
15	66,000,000	520,000	7,900	60,000,000	1,100,000	18,000
25	66,000,000	520,000	7,900	60,000,000	1,000,000	17,000
50	66,000,000	1,200,000	18,000	60,000,000	1,100,000	18,000

against coagulation so that even the plain agar is able to induce peptization, and hence the addition of sugar to the medium is of no special advantage.

Hypertonic Solutions in Milk.

Effect of plain and carbohydrate agar on apparent survival. Sterile milk was heavily inoculated with a pure culture of E. coli 57 and then diluted (1) with an equal volume of water, and (2) with an equal volume of 100 per cent sucrose, thereby giving an ultimate concentration of 50 per cent sugar. These two samples were plated before and after heating (54.5°C. 9 minutes) on plain, and on 1 per cent dextrose, sucrose, and lactose agars.

The results in table 5 show that approximately ten times as many organisms survived in the milk to which sucrose had been added. It is of interest to observe that the use of carbohydrate agar in this experiment did not increase the number of survivors. Such an observation tends to discourage the conclusion that the injured cells are necessarily rendered more saccharophilic.

Effect of prolonged contact of organisms with 50 per cent sucrose on thermal resistance. A tube of sterile milk was inoculated with E. coli 52 and incubated until it contained several million organisms per cc. The culture was then

Table 5. Effect of various media on the recovery of *E. coli* 57 heated (54.5°C., 9 minutes) in suspensions of milk, and milk containing 50 per cent sucrose.

Agar	Count per cc.					
	Before heating		After heating, 54.5°C., 9 minutes		Survival per million	
	No sugar	50 per cent sugar	No sugar	50 per cent sugar	No sugar	50 per cent sugar
Plain	10,000,000	10,000,000	480,000	2,500,000	48,000	250,000
1 per cent dextrose	14,000,000	11,000,000	270,000	2,500,000	20,000	230,000
1 per cent sucrose	10,000,000	11,000,000	220,000	2,700,000	18,000	245,000
1 per cent lactose	12,000,000	15,000,000	360,000	2,300,000	30,000	150,000

placed in iced water to retard growth. One cc. of this milk was placed in each of two tubes containing an equal volume of (1) sterile water and (2) sterile 100 per cent sucrose solution. These were thoroughly mixed and placed at room temperature for 27 hours. At appropriate intervals other tubes containing 0 and 50 per cent sucrose were prepared in a similar manner, so that at a given time pairs of tubes were available which had been exposed for 0, 3, 18, and 27 hours. These were plated on plain and lactose agar before and after heating at 54.5°C. for 5 minutes; one-tenth cc. samples were heated in the thin walled, blown tubes and submerged in a water bath. After heating, 9.9 cc. of sterile water were introduced for the first (1-100) dilution.

The data are presented in table 6 and shown graphically in figures 1 and 2. To facilitate comparison, the data of table 6 have been calculated to a basis of survival per million and presented in table 7. A study of the data suggests that these cells became more resistant to heat with prolonged exposure to the sugar. This was especially true with the sucrose suspension, although the actual percentage increase in resistance was greater in the suspension without sugar.

The general similarity of the curves in figures 1 and 2 shows that there was very little difference in the counts on plain and lactose agars. Prolonged exposure to 50 per

Table 6. The thermal resistance of E. coli 52 after prolonged exposure to sugar.

Hours contact before heating	No sugar				50 per cent sucrose			
	Count per cc.*				Count per cc.*			
	Before heating		After heating, 54.5°C., 5 minutes		Before heating		After heating, 54.5°C., 5 minutes	
	Plain agar	1 per cent lactose agar	Plain agar	1 per cent lactose agar	Plain agar	1 per cent lactose agar	Plain agar	1 per cent lactose agar
0	320,000	300,000	18	29	250,000	280,000	20,000	50,000
3	290,000	290,000	250	850	200,000	250,000	2,000	4,100
18	220,000	220,000	150	160	180,000	190,000	59,000	56,000
27	470,000	470,000	500	600	93,000	120,000	56,000	78,000

* 000 omitted from each value in this table.

Table 7. Data in table 6 calculated on a basis of survival per million after heating (54.5°C., 5 minutes).

Hours contact before heating	Survival per million in sample containing			
	No sugar		30 per cent sucrose	
	Plain agar	1 per cent lactose agar	Plain agar	1 per cent lactose agar
0	56	96	80,000	179,000
3	1,310	2,930	10,000	18,000
18	680	750	323,000	295,000
27	1,060	1,280	624,000	650,000

cent sucrose induced a decrease in numbers (lines C), although the numbers capable of withstanding the heat (54.5°C., for 5 minutes) increased (line D). The counts on the milk suspension before heating showed a decrease at first, followed by growth (line A). In this case, however, the slight increase in the number of survivors (line B) does not show well on the graph due to the scale of the drawing.

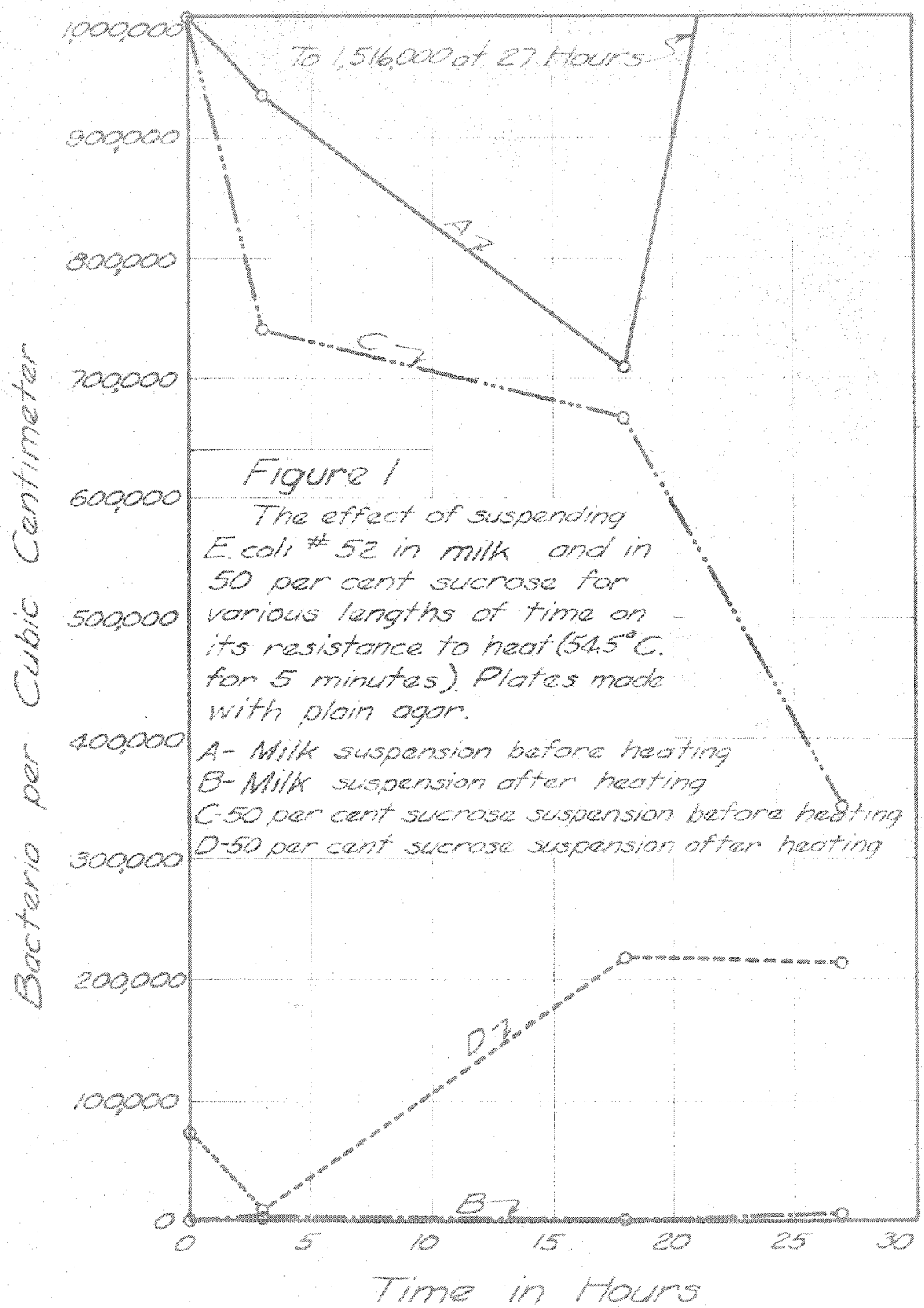

Figure 1

The effect of suspending E. coli # 52 in milk and in 50 per cent sucrose for various lengths of time on its resistance to heat (54.5° C. for 5 minutes). Plates made with plain agar.

A - Milk suspension before heating
B - Milk suspension after heating
C - 50 per cent sucrose suspension before heating
D - 50 per cent sucrose suspension after heating

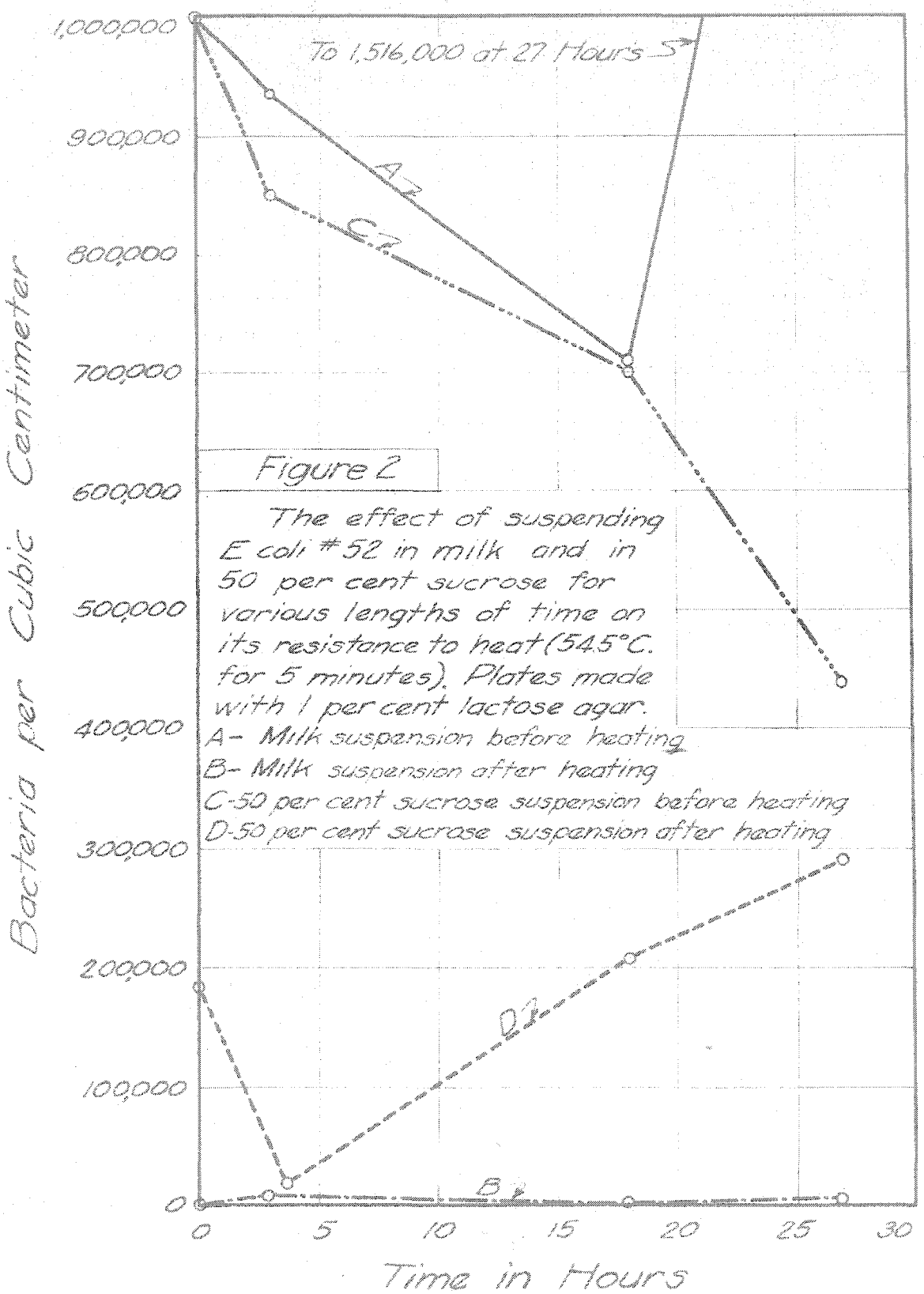

Figure 2

The effect of suspending E coli #52 in milk and in 50 per cent sucrose for various lengths of time on its resistance to heat (54.5°C. for 5 minutes). Plates made with 1 per cent lactose agar.

A- Milk suspension before heating

B- Milk suspension after heating

C-50 per cent sucrose suspension before heating

D-50 per cent sucrose suspension after heating

Hypertonic Solutions in Broth

In order to standardize the number of organisms in broth suspensions containing various percentages of sugar the following procedure was used. Double strength broth, containing 0.6 per cent beef extract and 1.0 per cent peptone was prepared, the reaction adjusted to pH 7.0, and the broth sterilized in 150 cc. quantities. A flask of this double strength broth was inoculated by adding 1 cc. of a broth culture of the desired organism. After thorough agitation to insure uniform distribution, equal portions (50 cc.) of this broth suspension of cells were transferred to flasks containing an equal volume of sterile water, or of sugar solutions of twice the ultimate percentage desired. In this way, approximately the same number of organisms were present in each concentration and the ingredients of the double strength broth were diluted to their usual percentage composition.

Protective action of sucrose in broth. Uniform suspensions of E. coli 52 in broth containing 0, 17, and 50 per cent sucrose were prepared by the method outlined. Ten, 5 cc. portions of each suspension were transferred to sterile test-tubes and heated in a water bath at 60°C. for 20 minutes, cooled, and plated on plain agar. The results given in table

8 show that cells heated in plain broth or broth containing 17 per cent sucrose were unable to survive heating at 60°C. for 20 minutes, whereas the presence of 50 per cent sucrose offered definite protective action.

Table 8. Survival of E. coli 62 after 20 minutes at 60°C. in broth containing 0, 17, and 50 per cent sucrose.

Count per cc. for the suspension of cells in					
Plain broth		17 per cent sucrose broth		50 per cent sucrose broth	
Before heating	After heating	Before heating	After heating	Before heating	After heating
7,000,000	0	12,000,000	0	6,000,000	216,000
7,000,000	0	12,000,000	0	6,000,000	376,000
7,000,000	0	12,000,000	0	6,000,000	385,000
7,000,000	0	12,000,000	0	6,000,000	350,000
7,000,000	0	12,000,000	0	6,000,000	349,000
7,000,000	0	12,000,000	0	6,000,000	362,000
7,000,000	0	12,000,000	0	6,000,000	706,000
7,000,000	0	12,000,000	0	6,000,000	200,000
7,000,000	0	12,000,000	0	6,000,000	312,000
7,000,000	0	12,000,000	0	6,000,000	271,000

In another experiment, after plating suspensions of E. coli 52 in plain and 50 per cent sucrose broth, ten 0.5 portions of each were transferred to small sterile tubes and hermetically sealed. These tubes had been prepared previously by blowing a bulb on one end of a piece of soft glass tubing (8 mm. bore) and drawing the other end out to a bore of about 2 mm. The sample was introduced by means of a special pipette and the tube quickly sealed. The 20 tubes were then completely submerged in a water bath held at 55°C. After 2 minutes exposure, duplicate tubes of both the plain and 50 per cent sucrose broth suspensions were removed and placed in cold water. Similarly, tubes were removed after 4, 6, 8, and 10 minutes exposure. The capillary end of each tube was flamed, and broken, then flamed again before emptying the contents of the tube into a sterile test-tube. A 0.1 cc. sample of the heated suspension was then plated on plain agar.

The results in table 9 show a material increase in the resistance of the cells in the 50 per cent sucrose broth suspension at all the exposures at 55°C. It is of interest to note that the count before heating in the 50 per cent sucrose broth was considerably less than that in the plain broth. This suggests that the sugar may have a destructive as well as a protective action.

Table 9. Thermal resistance of E. coli 52 suspended in broth and in 50 per cent sucrose broth.

Minutes exposure to 55°C.	Count per cc.		Survival per million	
	Plain broth	50 per cent sucrose broth	Plain broth	50 per cent sucrose broth
(Before heating)	2,000,000	750,000		
2	300	25,000	150	33,000
	*	14,000	*	18,700
4	30	7,500	15	10,000
	0	9,500	0	13,000
6	0	1,200	0	1,600
	0	650	0	870
8	0	300	0	400
	0	260	0	350
10	0	20	0	27
	0	50	0	66

* tube broken.

Comparison of the protective action of dextrose and maltose. Uniform suspensions of E. coli 52 in plain broth, 50 per cent dextrose broth, and 50 per cent maltose broth were prepared in the manner previously described. Five 0.5 cc. portions of each suspension were placed in sterile, narrow bore test-tubes (100 x 10 mm.). These were submerged to within 1.5 cm. of the top of the tubes in the oil bath at

54 ± 0.1°C. After 2, 4, 6, 8, and 10 minute exposures, one tube of each series was removed from the oil bath, placed in iced water, and subsequently plated on plain agar.

The comparative protective action of dextrose and maltose may be observed in the values for the survival per million given in table 10. It is evident that dextrose afforded some protective action to the cells but the numbers surviving in the maltose suspensions differed very little from those heated in plain broth. Even after 10 minutes exposure 115,000 per million of the cells suspended in dextrose broth survived, whereas none in the maltose or plain broth suspension was able to form colonies.

Effect of prolonged contact in hypertonic broth solutions on thermal resistance. The results of preliminary experiments had suggested the possibility that prolonged exposure to hypertonic solutions might increase the thermal resistance of cells.

A suspension of E. coli 52 in broth containing 35 per cent sucrose was distributed in 0.5 cc. portions in ten small tubes and the tubes hermetically sealed. After 1 hour at room temperature these were submerged in a water bath held at 54°C. One tube was withdrawn after each 1 minute interval, cooled in iced water and plated on plain agar. The original suspension was then kept at room temperature for 5 days when the same procedure was repeated.

Table 10. Survival of *E. coli* 52 when heated (54°C.) in plain broth, and in 50 per cent dextrose and maltose broths.

Minutes exposure to 54°C.	Count per cc.			Survival per million		
	Plain broth	50 per cent dextrose broth	50 per cent maltose broth	Plain broth	50 per cent dextrose broth	50 per cent maltose broth
0	470,000	200,000	280,000	-	-	-
2	51,000	48,000	44,000	109,000	240,000	157,000
4	2,700	28,000	800	5,750	140,000	2,860
6	200	37,000	100	425	185,000	360
8	20	36,000	20	42	180,000	714
10	0	23,000	0	0	115,000	0

The data are presented in table 11 and shown graphically in figure 8. Since the numbers of organisms increased about ten fold during the 5 days, the comparisons of the heat resistance can be made best by observing the values calculated on a basis of survival per million. Although the results show an increased number of survivors after aging for 5 days, they are not convincing because of the possible influence of the increased age and number of cells in the unheated sample.

Table 11. The thermal resistance of E. coli 52 after prolonged exposure to 25 per cent sucrose.

Minutes heated at 54°C.	Count per cc. after heating to 54°C.		Survival per million	
	Period of aging in 25 per cent sucrose		Period of aging in 25 per cent sucrose	
	1 hour	5 days	1 hour	5 days
0	33,000	4,700,000		
1	154,000	4,800,000	470,000	890,000
2	88,000	3,100,000	260,000	660,000
3	42,000	3,100,000	130,000	660,000
4	40,000	3,100,000	120,000	660,000
5	72,000	2,900,000	220,000	620,000
6	25,000	1,700,000	76,000	360,000
7	16,000	990,000	48,000	210,000
8	15,000		46,000	
9	9,000	1,000,000	27,000	210,000
10	4,600	43,000	14,000	9,000

In order to eliminate the possible influence of the age of the culture, cells were exposed to stronger solutions for

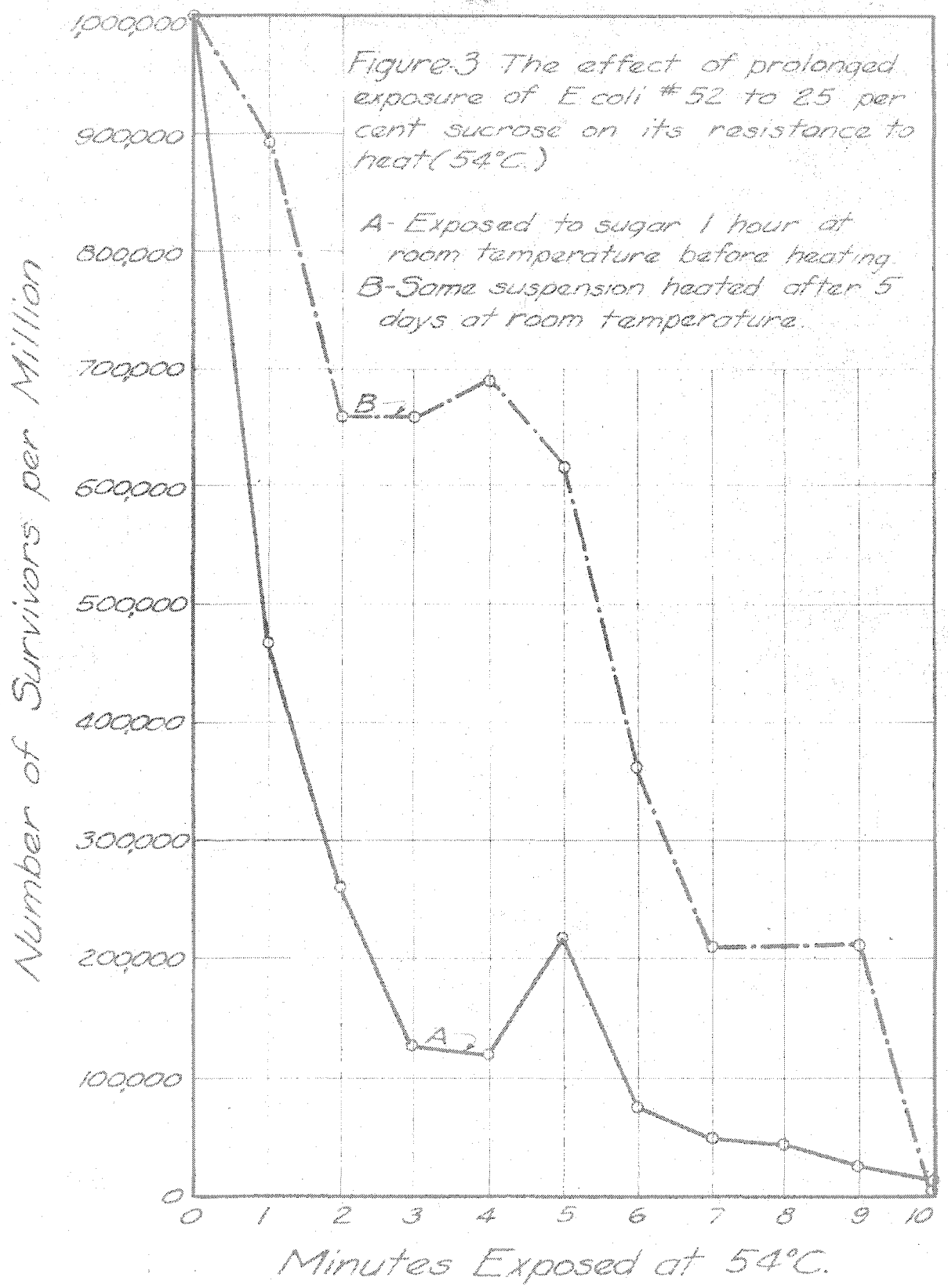

Figure 3 The effect of prolonged exposure of E coli # 52 to 25 per cent sucrose on its resistance to heat (54°C.)

A - Exposed to sugar 1 hour at room temperature before heating.
B - Some suspension heated after 5 days at room temperature.

shorter periods of time. Uniform suspensions of E. coli 25 were prepared in plain broth and in broths containing 50 per cent respectively of maltose, sucrose, and dextrose. Suspensions of cells in saturated lactose were inoculated separately. The suspensions were plated as quickly as possible on plain agar before and after heating to 54°C. for 9 minutes. After the original suspensions had stood at room temperature for 2 hours, plates were again made before and after heating to 54°C., for 9 minutes.

Table 12 shows that saturated lactose failed to protect E. coli 25 and that 50 per cent maltose broth afforded only very slight protection. It is of interest to note that the survival per million in 50 per cent sucrose and 50 per cent dextrose broth was increased seven to nine fold after aging 2 hours.

Tables 13 and 14 show the results of another experiment in which the same routine was followed except that the aging periods of 0, 2, and 48 hours were employed. As in the previous experiment, it is again evident that saturated lactose failed to protect the cells against heat. Suspension in 50 per cent maltose gave slight protection after prolonged contact. The results with sucrose are especially significant in that the actual number of organisms capable of surviving the heat treatment increased from 17,000 to 180,000

Table 12. Thermal resistance of *E. coli* 35 after 2 hours contact with plain broth, saturated lactose broth, and 50 per cent maltose, sucrose, and dextrose broths.

Cell suspension	Count per cc. after indicated time of contact				Survival per million (time of contact)	
	0		2 hours			
	Before heating	After heating 54.4°C., 9 min.	Before heating	After heating 54°C., 9 min.	0	2 hours
Plain broth	500,000	50	550,000	730	100	150
Saturated lactose broth	500,000	30	520,000	0	60	0
50 per cent maltose broth	500,000	1,100	120,000	280	2,200	2,900
50 per cent sucrose broth	240,000	9,800	220,000	58,000	29,000	264,000
50 per cent dextrose broth	600,000	18,000	60,000	13,000	30,000	217,000

Table 13. Thermal resistance of *E. coli* 25 after 3.5 and 48 hours contact with plain broth, saturated lactose broth, and 50 per cent maltose, sucrose, and dextrose broths.

Cell suspension	Count per cc.					
	Time of contact before heating					
	0		3.5 hours		48 hours	
	Before heating	After heating 54°C., 9 min.	Before heating	After heating 54°C., 9 min.	Before heating	After heating 54°C., 9 min.
Plain broth	1,300,000	0	7,200,000	0	5,600,000	0
Saturated lactose broth	2,000,000	0	1,960,000	0	2,000,000	7,000
50 per cent maltose broth	600,000	200	500,000	3,000	170,000	3,000
50 per cent sucrose broth	330,000	17,000	360,000	180,000	300,000	26,000
50 per cent dextrose broth	480,000	4,000	22,000	3,400	1,600	170

Table 14. Data from table 13 calculated to a basis of survival per million.

Cell suspension	Survival per million		
	Time of contact before heating		
	0	3.5 hours	48 hours
Plain broth	0	0	0
Saturated lactose broth	0	0	4
50 per cent maltose broth	300	10,000	52,000
50 per cent sucrose broth	45,000	500,000	87,000
50 per cent dextrose broth	8,700	150,000	110,000

per cc. during the 3.5 hour aging period (table 13), although there was no increase in the count of the unheated suspension. This suggests that the number of cells resistant to heat is increased with time although the hypertonic solution may be toxic in itself. There are likely two opposing tendencies, one a diminution of total numbers resulting from the high osmotic pressure and the other a stabilization of

the protoplasm against heat by physical changes induced. The more rapid disappearance of cells in the unheated dextrose suspensions may be due to the fact that this solution had about twice the osmotic pressure of the maltose and sucrose suspensions. Although the number of cells in the unheated dextrose suspension decreased from 460,000 to 82,000 during the 3.5 hour aging period, the number of cells capable of surviving the heat applied showed very slight change.

A study of the data in table 14 shows that the rate of increase in the thermal resistance during the first 3.5 hours aging was not maintained after 48 hours. This implies that during the first few hours of aging the thermal resistance of cells suspended in sucrose probably reaches a maximum. Accordingly an experiment was designed to study this point.

Suspensions of E. coli 52 in plain and 50 per cent sucrose broth were held at 30°C. Samples were removed from the sucrose suspension after each 15 minutes for 2 hours and then at less frequent intervals for 7 hours. Each sample removed was plated on plain agar before and after heating 54.5°C. for 5 minutes.

A study of the results presented in table 15 shows a marked increase in the thermal resistance during the first 2 hours. It is significant to note that although the counts

Table 15. The thermal resistance of *E. coli* 52 after prolonged incubation at 30°C. in plain and 50 per cent sucrose broth before heating to 54.5°C., 5 minutes.

Minutes contact before heating	Count per cc. in				Survival per million	
	Plain broth		50 per cent sucrose broth			
	Before heating	After heating, (54.5°C., 5 min.)	Before heating	After heating, (54.5°C., 5 min.)	Plain broth	50 per cent sucrose broth
0	1,500,000	10	1,700,000	42,000	7	25,000
15			1,700,000	170,000		100,000
30			1,800,000	450,000		250,000
45			2,200,000	570,000		259,000
60	2,600,000	0	2,300,000	750,000	0	326,000
75			1,700,000	870,000		512,000
90			1,800,000	960,000		533,000
105			1,700,000	690,000		406,000
120	4,100,000	0	1,400,000	1,100,000	0	786,000
180	53,000,000	0	2,900,000	550,000	0	190,000
240	150,000,000	0	1,200,000	350,000	0	292,000
300	220,000,000	10	1,100,000	210,000	0	191,000
420	330,000,000	0	930,000	57,000	0	61,000

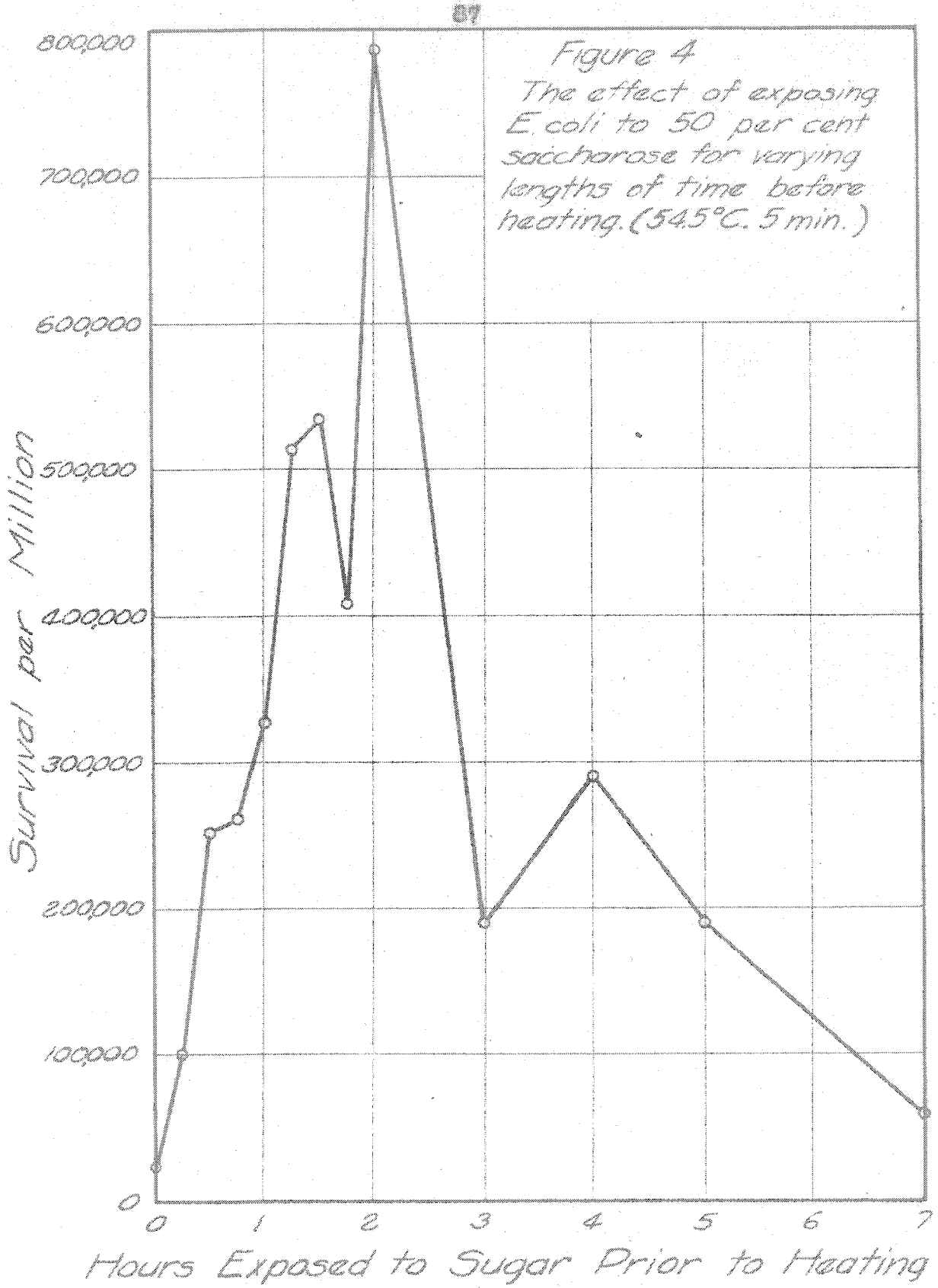

Figure 4
The effect of exposing E. coli to 50 per cent saccharose for varying lengths of time before heating. (54.5°C. 5 min.)

on the sucrose broth before heating were fairly constant, the actual number of cells capable of surviving the heat treatment increased. Evidently the physical changes responsible for greater heat stability of the protoplasm affects an increasing number of cells with time. In this instance the maximum number of cells capable of withstanding the heat treatment occurred after 2 hours exposure, whereas continued contact with the sugar resulted in a decrease in the thermal resistance.

The values for the survival per million in the 50 per cent sucrose broth have been plotted against time in figure 4. The graph clearly shows the tendency for the cells to become progressively more resistant to heat when exposed to hypertonic sucrose solutions for 2 hours and the tendency to lose this faculty on continued exposure.

As previously pointed out, the ability of the injured cell to recover depends upon the degree of coagulation induced and the peptizing qualities of the medium employed. High concentrations of sucrose induce a physical change, presumably resulting in the withdrawal of water at a rate which no doubt is regulated by the permeability of the individual cell. This process, although ultimately leading to the death of the cell, at first increases the stability of the protoplasm to heat. Continued exposure, however,

advances the degree of coagulation to a point beyond which plain agar can no longer induce peptization. The decline in the number of cells capable of surviving the heat treatment apparently is the result of prolonged desiccation beyond the point of optimum stability.

In order to further study the tendency of cells to become progressively more resistant to heat when exposed to hypertonic solutions, an experiment was devised to permit plating of the cells at more frequent intervals. E. coli 52 was suspended in 50 per cent dextrose broth and held at 30°C. Samples were removed and plated before and after heating at 54.4°C. for 5 minutes. Attention is called to the fact that this solution had an osmotic pressure approximately twice as great as a 50 per cent sucrose solution.

Table 18. Suspension of E. coli 52 in 50 per cent dextrose broth incubated at 30°C. for various lengths of time before heating, (54.4°C., 5 minutes).

Minutes contact before heating	Count per cc.		Survival per million
	Before heating	After heating, 54.4°C., 5 min.	
0	1,600,000	210,000	117,000
10	1,600,000	170,000	89,000
15	1,400,000	130,000	93,000
20	510,000	190,000	373,000
30	370,000	170,000	460,000
50	410,000	310,000	757,000
80	310,000	330,000	1,065,000
90	420,000	410,000	976,000
105	510,000	370,000	726,000
120	650,000	300,000	462,000

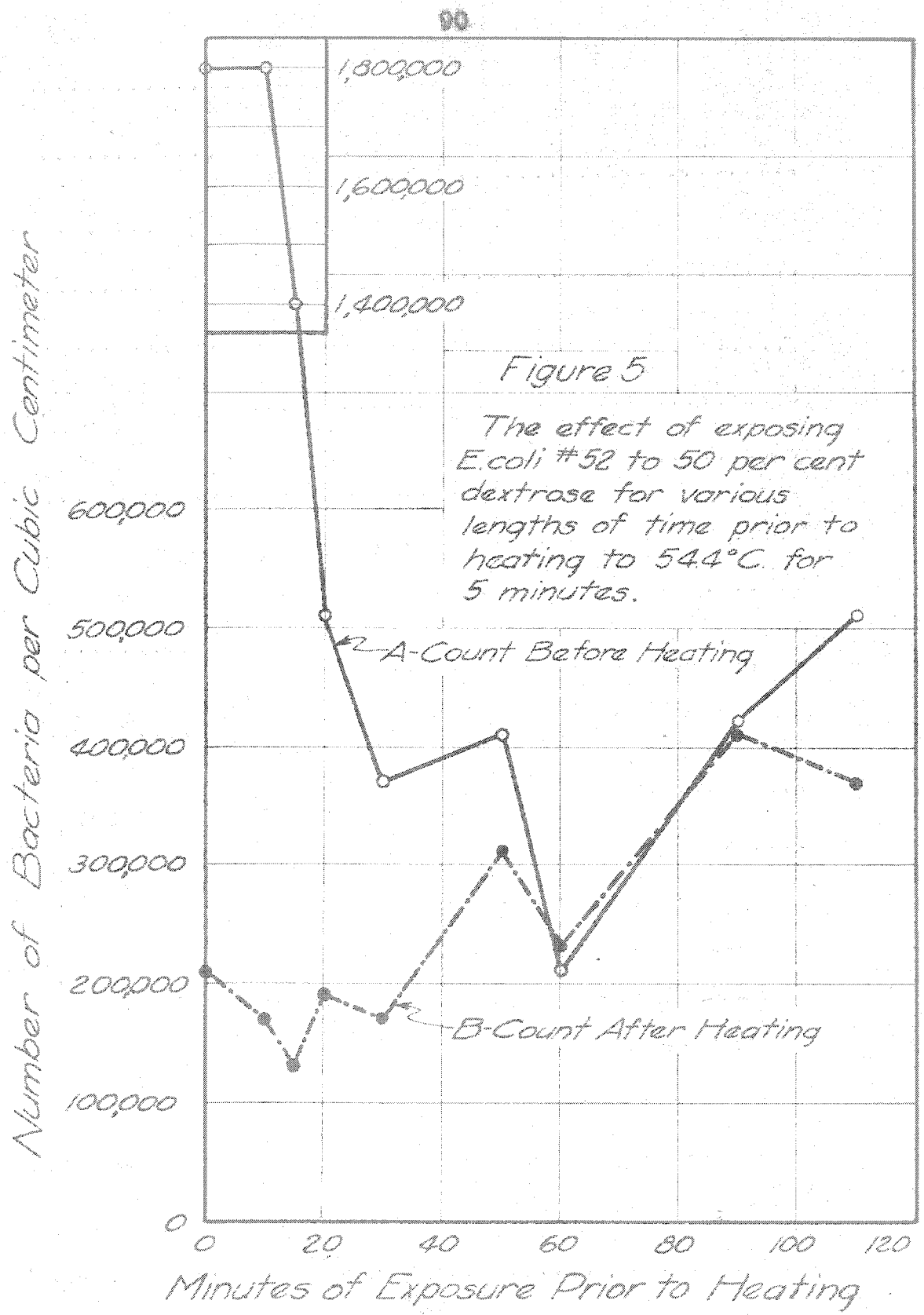

Figure 5

The effect of exposing E.coli #52 to 50 per cent dextrose for various lengths of time prior to heating to 54.4°C. for 5 minutes.

The data in table 16, arranged graphically in figure 5, illustrate the influence of the same factors demonstrated with sucrose suspensions. The same processes which are responsible for the rapid diminution of numbers in the unheated suspension induce greater thermal resistance in a progressively increasing number of cells.

Heat penetration. The broths containing high concentrations of sucrose and dextrose employed in previous experiments obviously were more viscous than the plain broth. No doubt the rate of heat penetration in such viscous solutions was less, and part of the increased survival observed in these suspensions may have been due to this factor. As pointed out in the description of the methods, small samples in special tubes were employed in heating trials to reduce the importance of this factor to a minimum. The relative rates of heat penetration in the various suspensions were frequently determined.

Five-tenth cc. portions of plain, and 50 per cent dextrose broth and also of a 100 per cent dextrose solution were placed in small bore (100 x 7 mm.) test-tubes. Standardized thermometers were inserted and the tubes first placed in cold water to render the temperatures of the three solutions uniform. They were then placed simultaneously in the oil bath at 54°C. Readings were taken at each 15 second

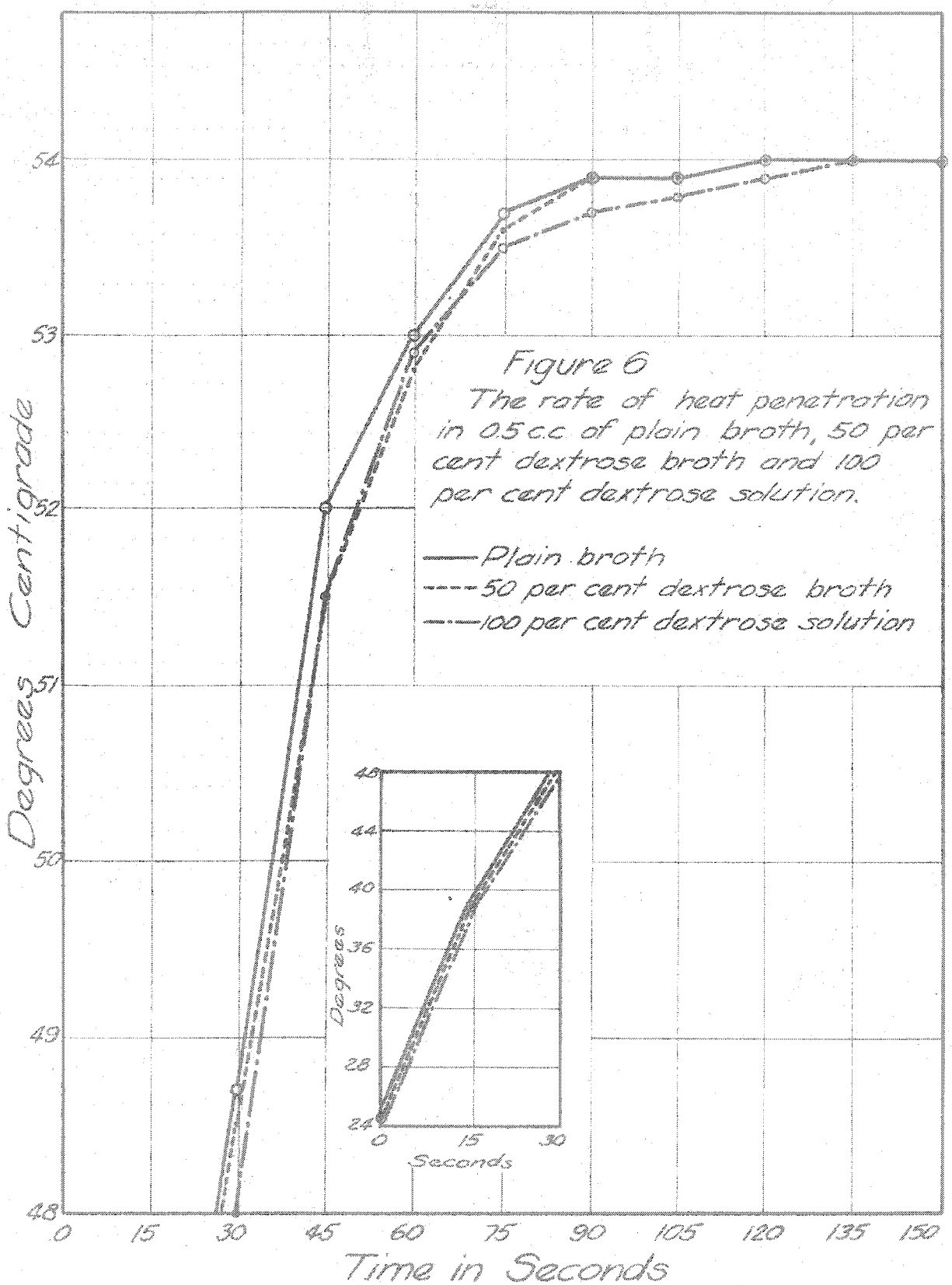

Figure 6

The rate of heat penetration in 0.5 c.c. of plain broth, 50 per cent dextrose broth and 100 per cent dextrose solution.

interval until the temperature had reached that of the oil bath. The temperatures increased so rapidly and at rates so nearly identical that it was difficult to make very accurate readings of the thermometers.

The graphs in figure 6 illustrate the very slight difference in the rates of heat penetration of the three solutions.

The peptizing qualities of plain and 1 per cent sucrose broth. A 48 hour plain broth culture of E. coli 52 was diluted 1-100 in water. Eleven plain broth and eleven 1 per cent sucrose broth tubes were inoculated with 0.1 cc. of this diluted culture and placed in a water bath at 54°C. One tube each of plain and sucrose broth was removed and cooled in iced water after 1, 2, 3, 4, 5, 6, 7, 8, 9, 12, and 20 minutes exposure to heat. The plain broth tubes were labeled the "A" series, and the 1 per cent sucrose broth tubes the "B" series. Immediately after cooling, a sub-culture was made from each tube to plain broth and another to 1 per cent sucrose broth. This provided suspensions of cells heated in plain broth and sub-cultured in plain and in 1 per cent sucrose broth ("A" series), and similarly, suspensions heated in 1 per cent sucrose broth transferred to plain and to sucrose broth ("B" series). All tubes, including those heated, were incubated at 37°C. for 48 hours.

Table 17. The thermal resistance of E. coli 52 in plain broth and 1 per cent sucrose broth.

Medium	Minutes exposure at 54°C.										
	1	2	3	4	5	6	7	8	9	12	30
"A" series. Heated in plain broth	+	+	+	+	+	-	-	-	-	-	-
Sub-culture from "A" series to plain broth	+	+	+	+	-	-	-	-	-	-	-
Sub-culture from "A" series to 1 per cent sucrose broth	+	+	+	+	-	-	-	-	-	-	-
"B" series. Heated in 1 per cent sucrose broth	+	+	+	+	+	+	+	+	-	-	-
Sub-culture from "B" series to plain broth	+	+	+	+	+	-	-	-	-	-	-
Sub-culture from "B" series to 1 per cent sucrose broth	+	+	+	+	+	-	-	-	-	-	-

\+ indicates growth
\- indicates no growth

The data in table 17 show that growth obtained in the original tubes of the "A" series (heated in plain broth) up to and including 5 minutes exposure to 54°C. The sub-cultures from these tubes to plain and sucrose broth showed growth up to and including 4 minutes exposure. Failure of growth in the sub-cultures made from the tube heated 5 minutes was

probably due to the fact that the number of viable cells had been so greatly reduced that the two loopfuls transferred to each of the sub-cultures failed to introduce living cells. That is, the majority of the cells were coagulated to such a degree that the media employed were unable to peptize them.

The broth in the "B" series showed growth after 8 minutes exposure, but the sub-cultures from tubes heated longer than 5 minutes failed to show growth. These results are not convincing and offer only slight evidence to indicate that 1 per cent sucrose broth delays the coagulation of cells by heat.

The same routine was followed in another experiment except that 50 per cent sucrose broth was substituted in the "B" series, and the time intervals were slightly altered. Sub-cultures were made to plain and 1 per cent sucrose broth as in the preceding experiments.

It will be observed in table 18 that the plain broth tubes ("A" series) gave results which were comparable with those obtained with the 1 per cent sucrose broth ("B" series) in the preceding experiment. This would tend to nullify any suggestion of increased thermal resistance afforded by 1 per cent sucrose broth in the preceding experiment. The results obtained with the cells heated in 50 per cent sucrose broth

show no growth in the original tubes due to the high osmotic pressure. The cells however were still viable as is shown by the sub-culture made to plain and 1 per cent sucrose broth. Sub-cultures made from the tubes in this series indicate that viable cells were still present even after 24 hours. These results are in harmony with what would be expected on a basis of the increased thermal resistance observed with the plating technique previously employed.

Table 18. The thermal resistance of E. coli 52 in plain broth and in 50 per cent sucrose broth.

	Minutes exposure at 54.4°C.									
	3	4	5	6	7	8	9	10	12	15
"A" series.										
Heated in plain broth	+	+	+	-	+	+	+	-	-	-
Sub-culture from "A" series to plain broth	+	+	+	+	-	-	-	-	-	-
Sub-culture from "A" series 1 per cent sucrose broth	+	+	+	-	-	-	-	-	-	-
"B" series.										
Heated in 50 per cent sucrose broth	-	-	-	-	-	-	-	-	-	-
Sub-culture from "B" series to plain broth	+	+	+	+	+	+	+	+	-	+
Sub-culture from "B" series to 1 per cent sucrose broth	+	+	+	+	+	+	+	+	+	+
Sub-culture from "B" series to 1 per cent sucrose broth after 24 hours	+	+	+	+	+	+	+	+	+	+

\+ indicates growth
\- indicates no growth

Hypertonic Solutions in Water

Effect of variations in osmotic pressure. An examination of the data in previous experiments shows that cells died more rapidly in unheated dextrose suspensions than in similar solutions of sucrose. The greater osmotic pressure of the dextrose solution at once suggests itself as a probable cause for this increased rate of death.

In order to study the influence of osmotic pressure on the thermal resistance of cells, E. coli 52 was suspended directly in hypertonic solutions of dextrose and sucrose of various molalities. One-tenth cc. of an 18 hour plain broth culture was placed in 5 cc. quantities of 4, 3, 2, and 1-molal dextrose, and in 8, 6, 4, 3, 2, and 1-molal sucrose solutions. After thorough agitation these were allowed to stand at room temperature for 2 hours. Two cc. portions of each suspension were then heated to 55°C. for 5 minutes.

The results of platings on plain and dextrose agar before and after heating are given in table 19. To facilitate comparison the data have been calculated to a basis of survival per million and presented in table 20. Due to the fact that each solution was inoculated separately, there was some unavoidable variation in the initial numbers of organisms in the solutions.

Table 19. A comparison of the protective action which equimolal solutions of dextrose and sucrose afford E. coli 52 when heated to 55°C. for 5 minutes.

Molality of sugar solutions	Sugar	Count per cc.			
		Before heating		After heating 55°C., 5 minutes	
		Plain agar	Dextrose agar	Plain agar	Dextrose agar
1	Dextrose	170,000	170,000	10,000	18,000
	Sucrose	1,500,000	2,200,000	400	15,000
2	Dextrose	740,000	720,000	11,000	14,000
	Sucrose	1,280,000	500,000	6,000	20,000
3	Dextrose	1,400,000	1,300,000	2,800	36,000
	Sucrose	330,000	540,000	68,000	57,000
4	Dextrose	1,200,000	1,100,000	50	280
		200,000	510,000	47,000	45,000
6	Sucrose	40,000	65,000	14,000	13,000
8	Sucrose	45,000	70,000	14,000	31,000
0	Water	2,700,000	2,700,000	30	0

Table 20. The survival per million of *E. coli* 52 when heated in equimolal solutions of dextrose and sucrose. Values calculated from data in table 19.

Agar	Survival per million in							
	4-molal		3-molal		2-molal		1-molal	
	Dextrose	Sucrose	Dextrose	Sucrose	Dextrose	Sucrose	Dextrose	Sucrose
Plain	41	225,000	1,900	310,000	15,000	26,000	59,000	2,700
1 per cent dextrose	2,400	139,000	29,000	124,000	20,000	41,000	106,000	6,800

Since the osmotic pressures of the equimolal solutions were identical the differences observed in the protective action cannot be accounted for on this basis. It is possible, however, that there was a difference in the effective osmotic pressure at the cell surface, since this would depend upon the permeability of the individual cell membrane. These results are in harmony with those of Cook (1931) to which reference has already been made.

In two other similar experiments, solutions were prepared containing 0.25, 0.50, 0.75, 1.0, 1.5, 2.0, 3.0, and 4.0-molal concentrations of dextrose and sucrose. Two cc. portions of each of these solutions, together with three controls, each containing 2 cc. of water, were placed in a water bath at 54.5°C. After these had reached the temperature of the water bath, a pair of the tubes containing equimolal concentrations of the respective sugars was removed, inoculated with 0.1 cc. of a 1-40 dilution of a 24 hour plain broth culture of E. coli 52, and immediately replaced in the water bath for 5 minutes exposure. This pair of tubes was cooled for 1 minute in iced water and immediately plated on plain and dextrose agars. A second pair of tubes, likewise containing equimolal concentrations, was then removed, inoculated, heated, and plated in a similar manner. The same process was followed with each pair of tubes. Since the experiment required about 2 hours, the 1-40 dilution of the culture was

Table 21. A comparison of the protective action which equimolal solutions of dextrose and sucrose afford E. coli 52 when heated to 54.5°C. for 5 minutes.

Molality of sugar solutions	Count per cc. after heating * 54.5°C., 5 minutes			
	Dextrose solutions		Sucrose solutions	
	Plain agar	Dextrose agar	Plain agar	Dextrose agar
0.25	0	0	20	0
0.50	0	50	0	20
0.75	0	70	300	2,200
1.0	10	40	1,200	11,000
1.5	600	1,300	19,000	25,000
2.0	1,700	1,700	29,000	36,000
3.0	5,200	2,400	30,000	35,000
4.0	16,000	11,000	16,000	21,000
0 (water)	50	10		
0 (water)	1,500	180		
0 (water)	190	0		

* Count per cc. before heating 110,000.

Table 22. The survival per million of E. coli 52 when heated in equimolal dextrose and sucrose solutions. Values calculated from data in table 21.

Molality of sugar solutions	Survival per million in			
	Dextrose solutions		Sucrose solutions	
	Plain agar	Dextrose agar	Plain agar	Dextrose agar
0.25	0	0	180	0
0.50	0	450	0	180
0.75	0	600	270	21,000
1.0	90	380	11,000	100,000
1.5	4,700	12,000	170,000	210,000
2.0	15,000	15,000	260,000	350,000
3.0	47,000	81,000	270,000	380,000
4.0	150,000	100,000	150,000	190,000

Table 23. A comparison of the protective action which equimolal solutions of dextrose and sucrose afford *E. coli* 52 when heated to 54.5°C., 5 minutes.

Molality of sugar solutions	Count per cc. after heating* 54.5°C., 5 minutes.			
	Dextrose solutions		Sucrose solutions	
	Plain agar	Dextrose agar	Plain agar	Dextrose agar
0.25	40	0	190	10
0.50	80	0	40	40
0.75	10	40	7,300	24,000
1.0	90	1,100	80,000	51,000
1.5	8,200	32,000	110,000	800,000
2.0	5,100	16,000	97,000	480,000
3.0	12,000	43,000	40,000	190,000
0 (water)	24,000	1,300		

* Count before heating = 3,300,000 per cc.

Table 24. The survival per million of *E. coli* 52 when heated in equimolal dextrose and sucrose solutions. Values calculated from data in table 23.

Molality of sugar solutions	Survival per million in			
	Dextrose solutions		Sucrose solutions	
	Plain agar	Dextrose agar	Plain agar	Dextrose agar
0.25	24	0	120	6
0.50	12	0	24	24
0.75	6	24	4,400	15,000
1.0	54	670	18,000	31,000
1.5	3,800	13,000	57,000	182,000
2.0	31,000	9,700	5,900	261,000
3.0	7,300	26,000	24,000	120,000

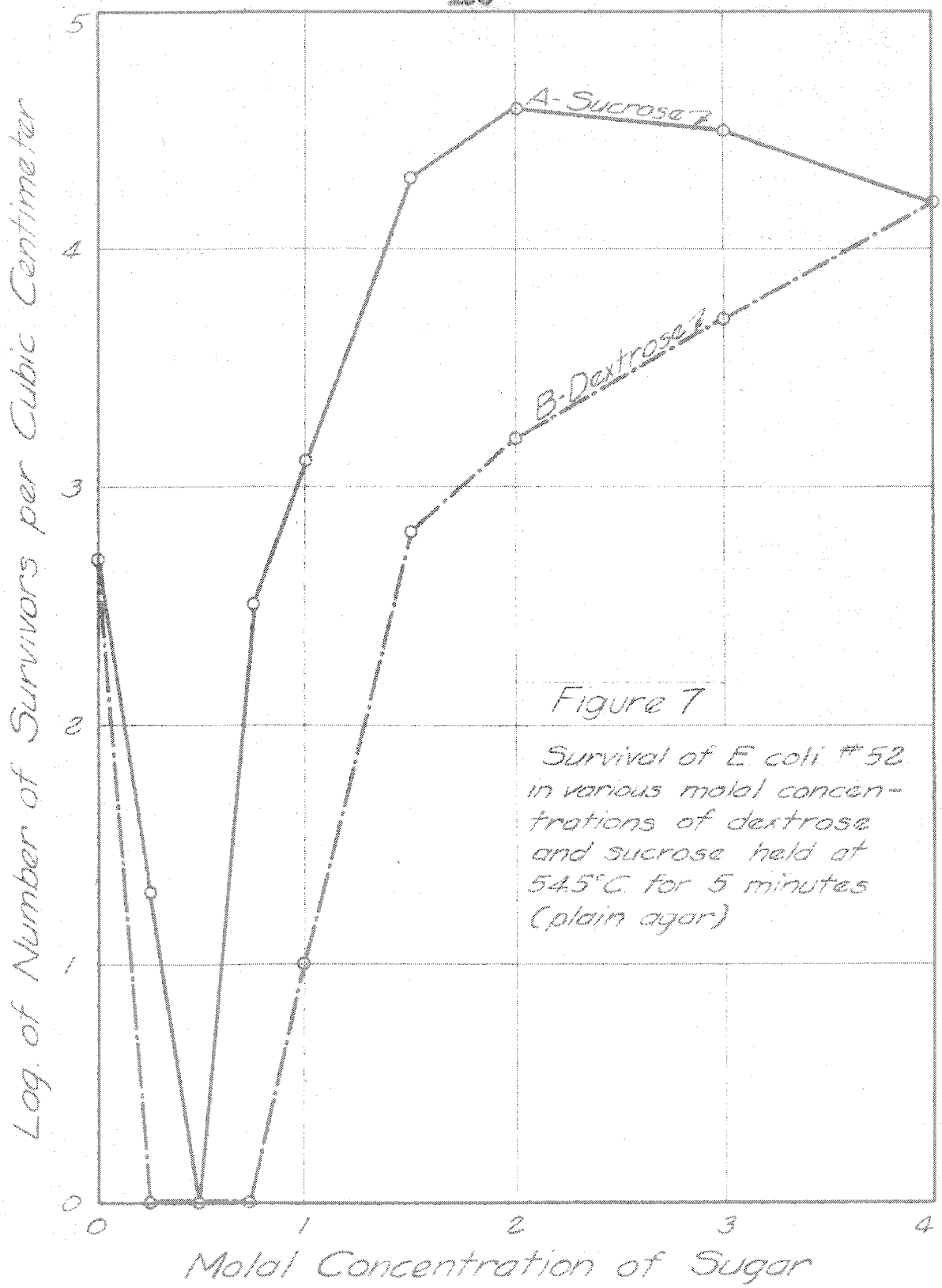

Figure 7

Survival of E coli #52 in various molal concentrations of dextrose and sucrose held at 54.5°C for 5 minutes (plain agar)

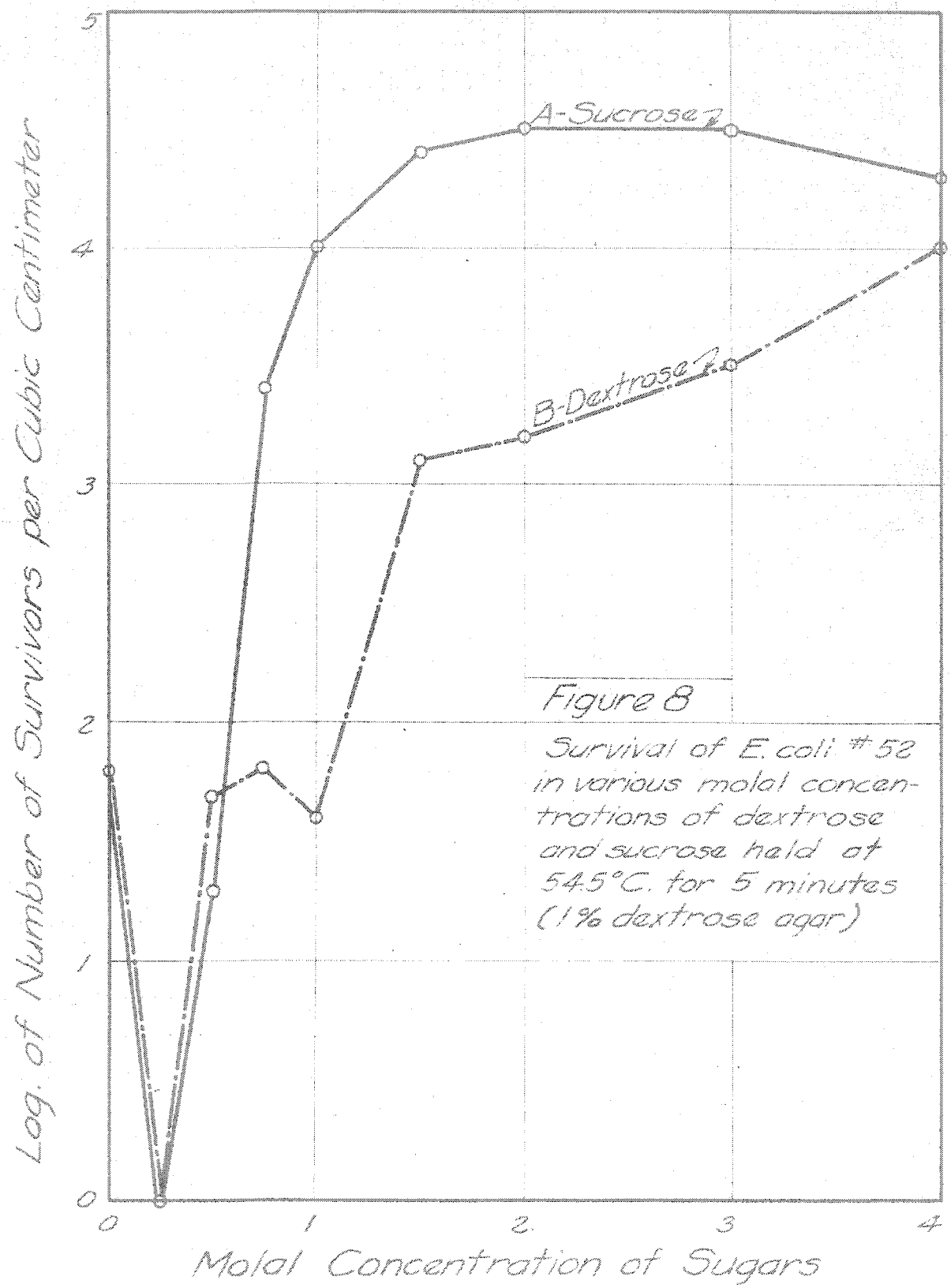

Figure 8

Survival of E. coli #52 in various molal concentrations of dextrose and sucrose held at 54.5°C. for 5 minutes (1% dextrose agar)

packed in ice to retard growth and to insure more uniform inoculation of the tubes at the beginning and end of the experiment. One of the water suspension controls was heated at the beginning of the experiment, one when half of the sugar suspensions had been heated, and one at the end of the experiment.

The results are given in tables 21, 22, 23, and 24. Figures 7 and 8 show graphically the results obtained with plain and dextrose agars respectively and are based on the data reported in table 21.

Comparison of the effect of the equimolal concentrations can be made most easily by consideration of the values for survival per million given in table 22. It is evident that very little protective action is afforded by either sugar in concentrations below 1-molal. In interpreting these results, however, it should be borne in mind that the time of contact of the cells with the solutions was limited to 5 minutes plus the time required for plating. If the results in this experiment are compared with those in the immediately preceding experiment it will be observed that similar molal concentrations exhibited considerably more protective action, due, presumably to the longer exposure (2 hours) before the heating trials were begun. The values in table 22, as well as the graphs in figures 7 and 8 illustrate quite forcibly that there is an increased protective action with the in-

creasing osmotic pressures beyond 1-molal concentrations. It is further evident from these data that the sucrose solutions exert more protective action than similar molal concentrations of the dextrose. This again emphasized the point that the osmotic pressure of a solution is not the only factor responsible for protective action. A contrast of the results reported in tables 20 and 22 on the relative protective action of 4-molal dextrose and sucrose brings out an interesting point. When the cells were exposed for 2 hours before heating (table 20) 4-molal sucrose afforded much more protection than 4-molal dextrose; when the time of action was reduced to 5 minutes (table 22) dextrose was as effective as sucrose in protecting cells against heat. The importance of the time factor in the relative protective action afforded by equimolal concentrations further suggests that penetrability plays an important role.

Effect of adding sugar after heating. In order to demonstrate more conclusively that the increased survival of cells heated in hypertonic sugar solutions was not attributable to the sugar carried over into the medium, the following experiment was designed. A 24 hour plain broth culture of E. coli 52 was diluted with an equal volume of water in one tube and with an equal volume of 100 per cent sucrose solution in another. Plain and dextrose agar plates were made from these two suspensions before heating to 54.5°C.

for 5 minutes. After heating, a 1 cc. portion of the sucrose suspension was diluted with an equal volume of a 50 per cent sucrose solution. Similarly two 1 cc. portions of the water suspension were diluted with equal volumes of water and 100 per cent sucrose solutions respectively. These were plated on plain and dextrose agars after allowing 1 minute for contact of the sugar solution added after heating. Cell suspensions were therefore available which had been heated, (1) in 50 per cent sucrose and suspended for 1 minute in a 50 per cent sucrose after heating, (2) heated in water and held in 50 per cent sucrose for 1 minute after heating, and (3) heated in water and retained in water after heating. If the cells suspended in sucrose surround themselves with a layer of sugar which serves as an intimate source of energy, stimulating development in the agar plate, one would expect the suspension to which sugar was added after heating to exhibit approximately the same survival as the cells heated in the presence of sugar.

The results in table 25 show that the addition of sugar after the cells had been heated in water failed to induce recovery from injury. In fact the values for survival per million were actually less than those observed in the water suspension. Two experiments were performed similar to the one just described, except that 0.85 per cent NaCl was substituted for water in the preparation of cell suspensions.

Table 25. The effect of adding 50 per cent sucrose to cell suspensions of *E. coli* 52 after heating (54.5°C., 5 minutes). Controls suspended in water.

Treatment of cells	Agar	Count per cc.		
		Before heating	After heating, 54.5°C., 5 minutes.*	Survival per million
Heated in 50 per cent sucrose, then equal volume of 50 per cent sucrose added after heating.	Plain	78,000,000	2,600,000	67,000
	Dextrose	80,000,000	5,700,000	146,000
Heated in water, then equal volume of 50 per cent sucrose added after heating.	Plain	190,000,000	300,000	3,200
	Dextrose	230,000,000	280,000	2,300
Heated in water, then equal volume of water added after heating.	Plain	190,000,000	2,100,000	22,000
	Dextrose	230,000,000	1,100,000	10,000

* Since the samples were diluted with equal volumes of sucrose solution or water after heating, the values in this column should be doubled. The values for survival per million have been adjusted accordingly.

Table 38. The effect of adding 50 per cent sucrose to cell suspensions of *E. coli* 52 after heating (55.2°C., 5 minutes). Controls suspended in 0.85 per cent NaCl.

Treatment of cells	Agar	Count per cc. Before heating	Count per cc. After heating, 55.2°C., 5 minutes.	Survival per million*
Heated in 50 per cent sucrose, then equal volume of 50 per cent sucrose added after heating.	Plain	140,000,000	52,000,000	371,000
	Dextrose	200,000,000	52,000,000	260,000
Heated in 0.85 per cent NaCl then equal volume of 50 per cent sucrose added after heating.	Plain	320,000,000	120	0
	Dextrose	270,000,000	0	0
Heated in 0.85 per cent NaCl then equal volume of saline added after heating.	Plain	320,000,000	7,900	49
	Dextrose	270,000,000	430	3

* The values in this column have been adjusted to account for the dilution of the sample after heating with equal volume of sucrose or saline solution.

Table 27. The effect of adding 50 per cent sucrose to cell suspensions of *E. coli* 52 after heating (55.2°C., 5 minutes). Controls suspended in 0.85 per cent NaCl.

Treatment of cells	Agar	Counts per cc. Before heating	Counts per cc. After heating, 55.2°C., 5 minutes.	Survival per million*
Heated in 50 per cent sucrose, then equal volume of 50 per cent sucrose added after heating.	Plain	66,000,000	14,000,000	424,000
	Dextrose	110,000,000	9,400,000	171,000
Heated in 0.85 per cent NaCl, then equal volume of 50 per cent sucrose added after heating.	Plain	170,000,000	0	0
	Dextrose	110,000,000	0	0
Heated in 0.85 per cent NaCl, then equal volume of saline added after heating.	Plain	170,000,000	0	0
	Dextrose	110,000,000	0	0

* The values in this column have been adjusted to account for the dilution of the sample after heating with equal volume of sucrose or saline solution.

The points of interest to be observed in the data presented in tables 26 and 27 are identical and will be discussed simultaneously. It is quite evident that the cells heated in sucrose solutions were more effectively protected against the action of heat than those heated in 0.65 per cent NaCl. It is significant to note that in both experiments, the addition of sucrose after heating failed to produce apparent survival. This suggests that the protective action of sugar is dependent upon its effect on the cell rather than its effect on the culture medium. The data in tables 25, 26, and 27 effectively eliminate the possibility that the increased survival in hypertonic suspensions might be due to the cell carrying over into the medium a film of the heavy syrup which would serve as an intimate source of energy for regeneration. Whatever damage has been done to the cells after heating in water apparently cannot be counteracted by the subsequent addition of sugar. If the sugar is present during the heating a relatively large number of the cells are protected from irreversible injury.

Effect of sub-lethal temperatures on the cells suspended in hypertonic solutions. Flasks containing 40 cc. each of 1-molal dextrose, 1-molal sucrose, and water were placed in a water bath at 44.5°C. until they had attained that temperature. One of these was removed, inoculated with 1.0 cc. of a 15 hour

broth culture of E. coli 52, thoroughly shaken, sampled for plating, and the flask immediately returned to the water bath. The plating was done as quickly as was consistent with care and accuracy, and in no instance did more than 2 minutes elapse between the time the organisms were introduced into the sugar solution and the completion of plating. The same procedure was followed for the other two flasks. After exposure to 44.5°C. for exactly 20, 30, 60, 90, and 120 minutes, the respective flasks were removed from the water bath and the suspension quickly plated on dextrose agar.

Table 28. The effect of exposing E. coli 52 to hypertonic solutions at a sub-lethal temperature (44.5°C.).

Minutes held at 44.5°C.	Count per cc. after suspension had been held at 44.5°C. in*		
	1-molal dextrose	1-molal sucrose	Water
0	5,600,000	4,800,000	7,600,000
20	780,000	1,400,000	7,800,000
30	320,000	1,500,000	------
60	220,000	1,200,000	7,700,000
90	130,000	1,800,000	------
120	55,000	980,000	8,200,000
180	31,000	760,000	8,000,000

* Number of cells originally introduced = 9,000,000 per cc.

The results in table 28 show that the organisms suspended in water were not destroyed in 3 hours, but that there was a very rapid reduction in numbers in both sugar solutions. Although the two solutions had the same osmotic pressure the rate of destruction in the dextrose was considerably greater than in sucrose. This was especially noticeable after the 20 minute observations. Apparently there was a rather marked destruction of the more sensitive cells in both solutions in the first few minutes of exposure. This destruction continued at a less rapid rate in the sucrose suspension. The probable relation between the destructive tendencies and the relative protective properties of these sugars presents an interesting point. If there is a relationship between the rate of destruction of cells in hypertonic solutions and the protective action, it suggests that the two processes may be the result of the same set of forces. Such observations would suggest that exposure to hypertonic solutions ultimately leads to the death of the cell, probably by the withdrawal of water and coagulation of the protoplasm. In proceeding from a condition of optimum colloidal dispersion of the cell ingredients to irreversible coagulation, the protoplasm passes through a stage of increased stability. The opportunity to observe this zone depends upon the speed of the major process leading to ultimate coagulation. For sugars

such as dextrose, which induce rapid destruction of the cells, the zone of increased stability is narrow, whereas the slower rate of desiccation observed in the sucrose suspensions facilitates the detection of protective action.

In order to enable further comparison of the influence of osmotic pressure on the rate of death of cells at the lower temperatures, suspensions of a 15 hour plain broth culture of E. coli 52 were made in water, 1-molal and 3-molal solutions of dextrose and sucrose. The same technique of heating and of plating employed in the preceding experiment were used except that the time intervals were extended.

The data are presented in table 29 and are shown graphically in figure 9. In this experiment, the water suspension was inadvertently destroyed after the 4.5 hour observation so that no data are available for 9 and 24 hours. It is apparent, however, that there was no diminution of numbers in the water as far as the observations were available.

The counts obtained when suspensions were plated immediately after inoculation indicate a very rapid reduction in numbers during the very short interval (less than 3 minutes) required for dilution and plating. This is especially noticeable in the 3-molal dextrose suspension, in which only 300,000 of the expected 6,000,000 cells were able to grow.

The marked difference in the rate of death in the various suspensions is illustrated by the observation that cells

Table 29. The effect of exposing E. coli 52 to hypertonic solutions at a sub-lethal temperature (44.5°C.).

Hours held at 44.5°C.	Count per cc. after suspensions had been held at 44.5°C. in *				
	1-molal dextrose	3-molal dextrose	1-molal sucrose	3-molal sucrose	Water
0	3,900,000	300,000	1,600,000	1,400,000	4,000,000
0.5	2,000	0	1,200,000	150,000	3,500,000
1.0	0	0	310,000	13,000	5,900,000
2.0	0	0	250,000	4,000	5,100,000
4.5	0	0	230,000	80	4,800,000
9.0	0	0	130,000	0	-
24.0	0	0	270	0	-

* Number of cells originally introduced = 5,250,000 per cc.

Figure 9
Survival of E. coli #52 in various molal concentrations of dextrose and sucrose, and in water when held at 44.5°C.
A- Water
B-1-Molal Sucrose
C-3-Molal Sucrose
D-1-Molal Dextrose
E-3-Molal Dextrose
Hours of Exposure
Log. of Survivor per Cubic Centimeter
0
1
2
3
4
5
6
7
8
9
22

died more rapidly in 1-molal dextrose than in 3-molal sucrose. On the other hand a comparison of the data obtained from 1-molal and 3-molal sucrose suggests that the factor responsible for diminution in numbers is increased with higher osmotic pressures.

The essential features of the data are more clearly presented in figure 9. It is apparent from the trend of curve A that the cells were not destroyed in water at 44.3°C. Comparison of curves B and D, and of C and E shows that the organisms were more sensitive to dextrose than sucrose in equimolal concentrations. The trends of these curves also indicate that the osmotic pressure of a given substance influences the rate of destruction, but that the same osmotic pressures may not induce the same rate of destruction when another substance is employed. This, in turn, suggests that the semipermeability of the cell membrane plays an important role in regulating the phenomena under investigation.

The results of the two preceding experiments prompted the plan of comparing the relative destruction of E. coli 52 in water, 1-molal dextrose, and 1-molal sucrose held at still lower temperatures. Accordingly, such cell suspensions were prepared and held at 37°C., 31°C., 21°C., and 6°C. In addition to the plan followed in the two preceding experiments, samples were removed from the three suspensions at intervals and heated to determine the relative thermal resistance

of the cells. Since the same technique was employed in obtaining all of the data in tables 30, 31, 32, and 33 a single outline of the procedure will suffice.

Three flasks containing 40 cc. of water, 1-molal dextrose, and 1-molal sucrose respectively were placed at the temperature chosen for incubation (37°C.---6°C. etc.) and held until ample time had been allowed to attain the desired temperature. One of the flasks was removed, inoculated with 1 cc. of a 13-16 hour plain broth culture of E. coli 52, vigorously shaken, 2 cc. transferred to a sterile tube for heating, 1 cc. removed for plating, and the flask quickly returned to the incubator. The sample reserved for heating was immediately placed in a water bath at 54.5°C. for 5 minutes, removed to iced water for 30 seconds. The plating before and after heating was done rapidly and with rather carefully standardized speed. Due to the necessity for rapidity in plating, only single plates of dextrose agar were prepared from each dilution. The two remaining flasks were inoculated and the contents heated and plated by the same procedure.

After the suspensions had been held for the indicated length of time, samples were again removed for plating and heating as before. The intervals between observations were not uniform for the various temperatures employed but were arranged according to the expected rates of activity.

Table 30. The effect of exposing E. coli 52 to hypertonic solutions at a sub-lethal temperature (37°C.) on its thermal resistance when subsequently heated to 54.5°C., 5 minutes.

Minutes held at 37°C. before heating	Count per cc. in 1-molal dextrose *		Minutes held at 37°C. before heating	Count per cc. in 1-molal sucrose*		Minutes held at 37°C. before heating	Count per cc. in water*	
	Before heating	After heating, 54.5°C. 5 minutes.		Before heating	After heating, 54.5°C. 5 minutes.		Before heating	After heating, 54.5°C. 5 minutes.
0	6,500,000	7,700	0	6,400,000	800,000	0	12,000,000	0
11	1,800,000	6,400	25	1,100,000	65,000	20	10,000,000	20
21	870,000	3,700	56	870,000	76,000	50	7,500,000	0
30	360,000	3,400	150	830,000	71,000	135	12,000,000	0
45	120,000	2,200	330	560,000	49,000	330	55,000,000	0
60	65,000	2,000	660	340,000	55,000	654	72,000,000	10
120	22,000	550	1,440	760,000	40,000	1,440	100,000,000	15
225	6,700	100						
405	470	0						
720	650	0						
1440	150,000	1,200						

* Original number of cells introduced = 10,000,000 per cc.

Table 31. The effect of exposing E. coli 52 to hypertonic solutions at a sub-lethal temperature (31°C.) on its thermal resistance when subsequently heated to 54.5°C., 5 minutes.

Minutes held at 31°C. before heating	Count per cc. in					
	1-molal dextrose		1-molal sucrose		Water	
	Before heating	After heating, 54.5°C., 5 minutes	Before	After heating, 54.5°C., 5 minutes	Before heating	After heating, 54.5°C., 5 minutes
0	13,000,000	400,000	10,000,000	2,400,000	13,000,000	0
30	5,200,000	160,000	10,000,000	1,100,000	15,000,000	0
60	2,800,000	220,000	9,500,000	940,000	17,000,000	0
180	950,000	210,000	6,700,000	1,100,000	37,000,000	0
270	520,000	95,000	5,500,000	1,300,000	75,000,000	0
360	300,000	87,000	4,400,000	820,000	100,000,000	0

Table 38. The effect of exposing E. coli 52 to hypertonic solutions at a sub-lethal temperature (21°C.) on its thermal resistance when subsequently heated to 54.5°C., 5 minutes.

Hours held at 21°C. before heating	Count per cc. in					
	1-molal		1-molal sucrose		Water	
	Before heating	After heating, 54.5°C., 5 minutes.	Before heating	After heating, 54.5°C., 5 minutes.	Before heating	After heating, 54.5°C., 5 minutes.
0	10,000,000	1,100	10,000,000	31,000	7,000,000	0
0.5	920,000	1,900	3,500,000	43,000	7,400,000	0
1.0	690,000	3,700	3,900,000	15,000	7,000,000	0
2.0	180,000	2,300	1,600,000	71,000	6,000,000	0
6.0	100,000	220	520,000	5,400	30,000,000	0
8.0	1,200	30	58,000	660	35,000,000	0
24.0	4,800	0	180,000	60	58,000,000	0

Table 35. The effect of exposing E. coli 52 to hypertonic solutions at a sub-lethal temperature (6°C.) on its thermal resistance when subsequently heated at 54.5°C., 5 minutes.

Time held at 6°C. before heating	Count per cc. in					
	1-molal dextrose		1-molal sucrose		Water	
	Before heating	After heating, 54.5°C., 5 minutes.	Before heating	After heating, 54.5°C., 5 minutes.	Before heating	After heating, 54.5°C., 5 minutes.
0	7,500,000	10,000	6,100,000	860,000	6,100,000	0
1 hour	6,600,000	18,000	6,000,000	310,000	6,000,000	0
3 hours	1,500,000	22,000	1,600,000	110,000	7,400,000	0
8 hours	630,000	10,000	2,600,000	110,000	8,800,000	0
24 hours	390,000	5,600	1,900,000	70,000	8,500,000	0
32 hours	330,000	5,300	1,200,000	78,000	10,000,000	0
2 days	220,000	3,700	780,000	98,000	8,700,000	0
3 days	120,000	2,400	770,000	42,000	7,100,000	0
4 days	46,000	760	480,000	28,000	5,000,000	0
5 days	27,000	670	430,000	29,000	3,900,000	0
6 days	18,000	130	340,000	10,000	3,000,000	0
7 days	8,100	90	180,000	6,000	1,600,000	0
9 days	2,600	18	160,000	4,000	7,500,000	0

Table 30 shows the effect of holding suspensions of E. coli 52 in 1-molal dextrose, 1-molal sucrose, and in water at 37°C. on the rate of destruction of the cells, and also on the thermal resistance to 5 minutes exposure at 54.5°C. It will be observed here, as in many previous experiments, that the rate of destruction of cells is much more rapid in the dextrose suspensions than in equimolal sucrose. Also in harmony with results of previous experiments is the observation that although the cells suspended in water are able to grow they are very sensitive to heat. It is especially significant that continued exposure to 37°C. with its attendant destruction of cells in the sugar suspensions gave no evidence of an actual increase in the number of cells capable of withstanding exposure to 54.5°C.

In table 31 are presented the results of a similar experiment in which the suspensions were held at 31°C. As might be expected the rates of destruction of cells at 31°C. are very much slower in both sugar solutions than at 37°C. The same observations in the preceding experiment regarding the greater toxicity of dextrose than sucrose for the unheated cells, the greater protective action of sucrose than dextrose for the heated cells, and the extreme sensitivity to heat in water may be made in this experiment.

The same observations are outlined above are likewise applicable to the data presented in tables 32 and 33 which show the results of holding the suspensions at 21 and 6°C. respectively. Only in table 32 is there any evidence of an increase in the actual number of cells capable of resisting a given heat treatment. It will be observed in this table that after 2 hours at room temperature the cells in the sucrose suspension showed a greater thermal resistance than at previous or subsequent observations. Although this value (71,000) is about twice the number capable of surviving the same exposure when the suspension was first prepared, it is far from convincing. It is possible that more frequent observations would have definitely established this point.

When the cell suspensions were held at 6°C. (table 33), the rates of action were very greatly reduced. Had there been any tendency for an increase in the number of cells capable of withstanding the heat treatment, one would expect some evidence of it in the results in table 33. In this experiment the intervals between observations were somewhat long so that there still remains the possibility that a zone of maximum stability escaped detection.

Effect of hypertonic solutions on the thermal resistance of various bacteria. In most of the experiments reported thus far in this paper a culture designated as E. coli 52 was used, which was selected at random from among several strains of

E. coli in the stock cultures of the Bacteriology Department, Kansas Agricultural Experiment Station. The results already presented have suggested quite definitely that the permeability of the individual cell membrane plays an important part in the phenomenon of protective action. One should expect therefore that many bacteria would not be protected by hypertonic solutions.

A survey of the results of the experiments presented suggests that the protective action of dextrose and sucrose solutions is best demonstrated when the organisms are sensitive to heating in water. Pure cultures of several organisms were heated in suspensions of water, 2-molal dextrose, and 2-molal sucrose. Although each culture represented a separate experiment the routine procedure was the same in each case except for the temperature employed.

One cc. of an 18 hour plain broth culture of the test organism was inoculated into each of three flasks containing 40 cc. of water, 2-molal dextrose, and 2-molal sucrose respectively. After thorough agitation a sample was removed for plating, also 1.4 cc. transferred to a small (10 x 100 mm.) tube for heating. The time of heating was 5 minutes in all cases, but the temperature employed varied with the different organisms.

The data in table 34 are interesting in that they show

Table 34. The effect of hypertonic solutions on the thermal resistance of several cultures.

Culture	Count per cc. before heating in			Temperature C°. of exposure for 5 min.	Count per cc. after heating in		
	Distilled water	2-molal dextrose	2-molal sucrose		Distilled water	2-molal dextrose	2-molal sucrose
Serratia marcescens	28,000,000	25,000,000	17,000,000	55	100	540,000	160,000
Staphylococcus albus	40,000	16,000	60,000	55	0	0	0
Pseudomonas fluorescens	80,000	1,000	12,000	55	10	60	9,000
Aerobacter aerogenes	12,000,000	14,000,000	10,000,000	60	2,800	35,000	75,000
Staphylococcus aureus	13,000,000	37,000	7,900,000	60	120	190	2,600
Bacillus subtilis	130,000	130,000	140,000	90	20	130	500
Salmonella pullorum	1,000,000	1,500	1,000	55	190,000	1,100	1,200

striking variations in the effect of water, 2-molal dextrose and 2-molal sucrose on thermal resistance of cells. First it will be noted that there is little or no evidence that either dextrose or sucrose afforded these strains of Staphylococcus albus or Salmonella pullorum any resistance to heat. However, it should be noted that the counts before heating in the dextrose and sucrose suspensions of Salmonella pullorum are practically the same as those obtained after heating. When compared with the count on the aqueous suspension before heating it is evident that a very large percentage of the organisms died almost instantly when introduced into the sugar solutions, but that those which did survive were able to withstand the heat treatment.

There is only slight evidence that 2-molal sucrose protected B. subtilis, whereas the results with Pseudomonas fluorescens and Staphylococcus aureus indicate protective action more definitely. Serratia marcescens and Aerobacter aerogenes were unmistakably protected by the presence of either dextrose or sucrose. It is of interest to note that 2-molal dextrose apparently offered more effective protection to Serratia marcescens than did an equimolal solution of sucrose. The reverse was true, however, for Aerobacter aerogenes, that is the sucrose solution offered more effective protection against heat.

These results further emphasize the importance of the penetrability of the membrane of the individual cell as a factor in protective action. It should be noted that in each case in which protective action was observed the cell exhibited extreme sensitivity to heat in water suspensions.

The observations that sucrose or dextrose afford protective action for a certain species are limited to the strains employed. It should not be construed from these results that all cultures of Serratia marcescens will exhibit this phenomenon. Such generalizations are discouraged by the fact that strains of E. coli have been found which are not protected by the presence of sucrose as has been so frequently observed in the experiments with E. coli 52. This point is clearly brought out with the strains used in the following experiments on the effect of the source of the culture on thermal resistance.

Thermal Resistance of Cells Grown in Plain and Dextrose Broth.

If the cell membrane plays an important part in the protective action afforded by hypertonic solutions, it seemed logical to suspect that the source of the organism might affect the conditions at the cell surface. The following two

experiments were designed to permit comparison of the effect of growth in plain and dextrose broth on the thermal resistance in water and in hypertonic solutions. Incident to these observations the experiments demonstrate quite clearly the point mentioned in the preceding paragraph, namely, that not all cultures of E. coli are protected by the presence of sugar.

The experimental procedure followed in obtaining the data reported in tables 35 and 36 was identical in each case. The two experiments with the data will therefore be described, presented and discussed as a unit. E. coli 58 and E. coli 57 were each inoculated into plain and dextrose broth and incubated 20 hours at 37°C. The two cultures of each strain thus provided were each treated in the following manner. Two cc. of culture were transferred to a 99 cc. water blank in order to reduce the amount of broth carried over in the subsequent heating trial. This diluted culture was plated on plain and dextrose agar to give the count before heating. Two cc. of the diluted culture were transferred to a thin walled blown tube containing 2 cc. of sterile water. Another 2 cc. portion of the diluted culture was transferred to a similar tube containing 2 cc. of 100 per cent sucrose solution, thus reducing the concentration of sugar to 50 per cent. These were allowed to stand at room temperature for 1 hour,

Table 35. Comparison of the thermal resistance of E. coli 52 and E. coli 57 when grown in plain and dextrose broth and heated (56°C., 5 minutes) in 50 per cent sucrose and in water.

Culture E. coli No.	Source	Heating medium	Agar	Count per cc. Before heating	Count per cc. After heating, (56°C. 5 minutes)	Survival per million*
52	Plain broth	50 per cent sucrose	Plain	6,100,000	28,000	4,700
			Dextrose	5,000,000	32,000	6,400
		Water	Plain	6,100,000	0	0
			Dextrose	5,000,000	0	0
	1 per cent dextrose broth	50 per cent sucrose	Plain	5,400,000	53,000	9,800
			Dextrose	4,800,000	390,000	85,000
		Water	Plain	5,400,000	0	0
			Dextrose	4,800,000	0	0
57	Plain broth	50 per cent sucrose	Plain	4,500,000	22,000	4,900
			Dextrose	6,200,000	45,000	7,300
		Water	Plain	4,500,000	44,000	9,800
			Dextrose	6,200,000	60,000	9,700
	1 per cent dextrose broth	50 per cent sucrose	Plain	7,000,000	160,000	23,000
			Dextrose	11,000,000	520,000	47,000
		Water	Plain	7,000,000	1,200,000	172,000
			Dextrose	11,000,000	1,800,000	164,000

* These values are adjusted to account for dilution before heating.

Table 36. Comparison of the thermal resistance of E. coli 52 and E. coli 57 when grown in plain and dextrose broth and heated (55.5°C., 5 minutes) in 50 per cent sucrose and in water.

Culture E. coli No.	Source	Heating medium	Agar	Count per cc. Before heating	Count per cc. After heating, (55.5°C., 5 minutes)	Survival per million *
52	Plain broth	50 per cent sucrose	Plain	5,700,000	28,000	4,900
			Dextrose	7,800,000	40,000	5,100
		Water	Plain	5,700,000	0	0
			Dextrose	7,800,000	0	0
	1 per cent dextrose broth	50 per cent sucrose	Plain	8,300,000	760,000	92,000
			Dextrose	8,000,000	900,000	113,000
		Water	Plain	8,000,000	14,000	1,700
			Dextrose	8,300,000	2,200	275
57	Plain broth	50 per cent sucrose	Plain	4,400,000	80,000	18,000
			Dextrose	7,300,000	280,000	38,000
		Water	Plain	4,400,000	380,000	86,000
			Dextrose	7,300,000	420,000	58,000
	1 per cent dextrose broth	50 per cent sucrose	Plain	11,000,000	1,400,000	127,000
			Dextrose	10,000,000	2,600,000	260,000
		Water	Plain	11,000,000	2,500,000	227,000
			Dextrose	10,000,000	3,100,000	310,000

* These values are adjusted to account for dilution before heating.

after which the tubes were heated, cooled, and plated on plain and dextrose agars. For each strain of E. coli employed (52 and 57) the cells grown in plain broth were heated in water and also in 50 per cent sucrose then plated on plain and dextrose agars. Similarly the cells grown in 1 per cent dextrose broth were heated in water and in 50 per cent sucrose and plated on plain and dextrose agars.

Attention is called first to the results with E. coli 52 in tables 35 and 36. When heated in water suspensions this strain showed very little resistance; all of the cells were killed except in one instance (table 36) when the survival per million was 1700 on plain agar and 375 on dextrose agar. Growth in plain or dextrose broth did not materially affect the resistance to heat in water suspensions. In the 50 per cent sucrose suspensions, however, the cultures from dextrose broth showed considerably greater values for the survival per million after heating. In table 35 the plain broth culture showed values for the survival per million of 4,700 on plain, and 6,400 on dextrose agar, as compared to 9,600 on plain and 83,000 on dextrose agar for the culture grown in dextrose broth. Even more marked differences are noted in table 36. Referring again to the results of heating trials in 50 per cent sucrose, it will be observed that the values for the survival per million on plain and carbo-

hydrate agars were 4,900 and 5,100 for the cultures grown in plain broth; the corresponding values for the cultures grown in dextrose broth were 92,000 and 113,000.

The data relative to E. coli 57 likewise show rather marked increase of resistance for the cultures originally grown in dextrose broth. In table 35 the plain broth culture of E. coli 57 gave values for the survival per million ranging from 4,900 to 9,800, whereas in the dextrose broth culture 85,000 to 172,000 per million survived. In table 36 the corresponding values for plain broth cultures ranged from 18,000 to 86,000, whereas for dextrose broth cultures they ranged from 127,000 to 280,000.

These results with E. coli 57 emphasize even more than the results with E. coli 52 that the source of the organism may affect its thermal resistance. Perhaps the most striking observation in the results with E. coli 57 is that it is not as sensitive to heating in water as E. coli 52. In fact with the former strain most of the values for the survival per million are even higher for the water suspensions than for the 50 per cent sucrose suspensions. This is directly the reverse of the observations with E. coli 52. The results prove quite definitely that this strain of E. coli is not protected by sugar and that it is much more resistant to heat in water suspensions. This affords further sub-

stantiation of the suggestion offered by the results of many of the preceding experiments, namely that the cell membrane is an important factor in heat resistance and in regulating protective action.

The results reported in table 37 were obtained by the same procedure as outlined for the experiments reported in tables 35 and 36, except that the cells were heated in 50 per cent dextrose instead of 50 per cent sucrose. The only significant difference between these results and those observed in tables 35 and 36 is that when compared with the plain broth cultures, E. coli 52 did not exhibit the increased resistance when grown in dextrose broth and heated in 50 per cent dextrose. The observations made in tables 35 and 36 with respect to E. coli 57 may be confirmed in table 37. This culture was not so sensitive to heat in water and even showed significantly greater values for survival per million when heated in water than when heated in 50 per cent dextrose.

Age of Culture

It has been proved by many investigators that old cultures are more resistant than young cultures. This point was investigated with special reference to the possible effect which age of the culture might have on the protective effect of sugar.

Table 37. Comparison of the thermal resistance of *E. coli* 52 and *E. coli* 57 when grown in plain and dextrose broth and heated (55°C., 5 minutes) in 50 per cent dextrose and in water.

Culture *E. coli* No.	Source	Heating medium	Agar	Count per cc. Before heating	Count per cc. After heating, (55°C., 5 minutes)	Survival per million*
52	Plain broth	50 per cent dextrose	Plain	160,000	1,700	11,000
			Dextrose	200,000	3,400	17,000
		Water	Plain	160,000	0	0
			Dextrose	200,000	0	0
	1 per cent dextrose broth	50 per cent dextrose	Plain	6,600,000	2,700	410
			Dextrose	6,500,000	9,000	1,400
		Water	Plain	6,600,000	0	0
			Dextrose	6,500,000	0	0
57	Plain broth	50 per cent dextrose	Plain	900,000	29,000	2,700
			Dextrose	2,300,000	53,000	5,300
		Water	Plain	900,000	16,000	13,000
			Dextrose	2,300,000	16,000	7,000
	1 per cent dextrose broth	50 per cent dextrose	Plain	11,000,000	49,000	4,500
			Dextrose	10,000,000	55,000	5,500
		Water	Plain	11,000,000	450,000	41,000
			Dextrose	10,000,000	450,000	45,000

* These values are adjusted to account for dilution before heating.

Plain broth tubes were inoculated at intervals with E. coli 52 so that at a given time there would be available cultures which were 192,96, 48, 24, and 12 hours old respectively. The broth tubes to be used for the cultures were placed in the 37°C. incubator at the same time to insure the same degree of evaporation in all tubes. The cultures were inoculated serially, that is, the 96 hour culture was inoculated at the proper time from the 192 hour culture, the 48 hour culture from the 96 hour culture, etc.

One drop of culture representing each age was placed in 10 cc. of water and another drop (from the same pipette) was placed in 10 cc. of 100 per cent sucrose solution. After thorough agitation these were sampled for plating before heating, and 0.5 cc. portions placed in small bore tubes. The tubes were heated for 5 minutes at 54°C., cooled, and the contents plated on plain agar.

The results presented in table 58 show the highest value of survival per million for the 24 hour culture heated in sucrose. Although the lowest value 16,000 per million for the cultures heated in sucrose was obtained with the youngest culture, there is very little evidence in these data that the age of the culture exerted any very marked effect on the results.

Table 58. Effect of age of culture on resistance of *E. coli* 52 to 54°C., for 5 minutes.

Age of culture (hours)	Water suspension			100 per cent sucrose suspension		
	Count per cc.		Survival per million	Count per cc.		Survival per million
	Before heating	After heating		Before heating	After heating	
12	2,000,000	1,200	600	500,000	8,100	16,000
24	2,400,000	1,400	580	75,000	10,000	130,000
48	2,200,000	12,000	5,500	360,000	18,000	49,000
96	1,000,000	70	70	420,000	31,000	73,000
192	300,000	720	2,400	60,000	3,000	50,000

Thermal Resistance of Washed Cells.

In many of the experiments the results have indicated that the protective action afforded certain cells is intimately associated with the permeability of the cell membrane. Several experiments were devised in which attempts were made to alter the conditions at the cell surface by washing the cells.

Effect of washing cells before exposing to sugar solution. A 24 hour plain broth culture of E. coli 52 was centrifuged to throw down the cells, the supernatant broth was removed, and 10 cc. of 0.85 per cent NaCl added. After thorough agitation the cells were again thrown down by centrifugal means, the supernatant liquid removed and the cells washed with a second 10 cc. of NaCl solution. This was repeated until the cells had been washed four times. At the time of the last of these washings, another 10 cc. portion of the original broth culture was centrifuged, the supernatant broth removed, and the cells resuspended in 0.85 per cent NaCl. These were designated as unwashed cells although in reality they had been partially washed once by the technique of resuspension.

One cc. of the suspension of washed cells was transferred to a thin walled blown tube already containing an equal volume of 0.85 per cent NaCl. Another 1 cc. portion of the

washed cell suspension was transferred to another tube containing 1 cc. of 100 per cent sucrose. Similarly, a 1 cc. portion of the unwashed cell suspension was transferred to two tubes containing equal volumes of 0.85 per cent NaCl and 100 per cent sucrose respectively. After thorough agitation the four samples thus provided were plated on plain and dextrose agar. The tubes were then placed in the oil bath at 54.5°C. for 5 minutes, cooled in iced water 1 minute, and plated on plain and dextrose agars.

The data presented in table 39 show that when washed cells were heated in 0.85 per cent NaCl none survived, but when heated in 50 per cent sucrose, the survival was comparable to that observed with the unwashed cells. When the unwashed cells were heated in 0.85 per cent NaCl a very small number survived. These results suggest that the protective action which sugar affords the cell is not inhibited by any change at the cell surface induced by washing in 0.85 per cent NaCl.

The following experiment was designed to determine the effect of washing cells in water and in broth on their thermal resistance. The procedure outlined applies to both cultures.

Table 39. The effect of washing E. coli 52 on its thermal resistance when heated (54.5°C., 5 minutes) in the presence and absence of sugar, and plated on plain and dextrose agars.

Cells	Agar	0.85 per cent NaCl suspension		50 per cent sucrose suspension	
		Count per cc.		Count per cc.	
		Before heating	After heating, 54.5°C., 5 minutes	Before heating	After heating, 54.5°C., 5 minutes
Washed	Plain	19,000,000	0	4,600,000	360,000
	Dextrose	43,000,000	0	13,000,000	760,000
Not washed	Plain	100,000,000	1,200	240,000	350,000
	Dextrose	90,000,000	1,700	350,000	610,000

Table 40. The effect of washing *E. coli* 52 and 57 in water and in broth on their thermal resistance when heated (54.5°C., 6 minutes) in water and 2-molal sucrose suspensions.

Culture *E. coli*		Count per cc.			
		Cells washed in water		Cells washed in broth	
		Suspended in water	Suspended in 2-molal sucrose	Suspended in water	Suspended in 2-molal sucrose
52	Number of cells introduced	40,000,000	40,000,000	60,000,000	60,000,000
	Count before heating	54,000,000	130,000	57,000,000	8,300,000
	Count after heating	12,000,000	370	7,300,000	230,000
	Survival per million	300,000	9	122,000	3,600
57	Number of cells introduced	40,000,000	40,000,000	48,000,000	48,000,000
	Count before heating	32,000,000	14,000,000	37,000,000	41,000,000
	Count after heating	32,000,000	47,000	44,000,000	13,000,000
	Survival per million	800,000	1,200	920,000	270,000

Two 10 cc. portions of an 18 hour broth culture of E. coli 52 were centrifuged to throw down the cells. One of these was washed four times with sterile conductivity water, and the other was washed four times with broth, the cells then being suspended in water and broth respectively. One tenth cc. of the water suspension was transferred to each of two tubes containing 2.9 cc. of sterile conductivity water, and 2.9 cc. of 2-molal sucrose respectively. Similarly, 0.1 cc. portions of the broth suspension were transferred to tubes containing 2.9 cc. of sterile conductivity water and 2.9 cc. of 2-molal sucrose. After removing samples for plating before heating, these four tubes were placed in an oil bath at 54.5°C. for 6 minutes, removed to iced water 1 minute, and plated on dextrose agar. The same routine was followed for E. coli 57. For each culture there were available, therefore, samples which had been washed in water and heated either in water or in 2-molal sucrose solution; also samples which had been washed in broth and heated either in water or in 2-molal sucrose solution.

The data in table 40 show that washing E. coli 52 or E. coli 57 in either water or broth tended to reduce the resistance to 2-molal sucrose solution. The cells washed in water and heated in water were considerably more resistant to heat than comparable cells heated in 2-molal su-

crose suspensions. It is noteworthy that the cells washed in broth died more slowly when transferred to the sugar solution than did those washed in water.

Effect of washing cells after exposing to sugar. In the previous experiments cells were washed before they were exposed to sugar. In the following three experiments the cells were exposed to the action of sugar, then the sugar removed by washing and centrifuging.

Five cc. of an 18 hour plain broth culture of E. coli 52 were centrifuged and all but 1 cc. of the supernatant broth removed. One cc. of 100 per cent sucrose was added and the resulting 50 per cent sugar suspension of cells allowed to stand at room temperature for one-half hour. The cells were then washed seven times with 0.85 per cent NaCl solution to remove the sugar. At the appropriate time two 5 cc. portions of a broth culture of the same organism were centrifuged to concentrate the cells. All but 1 cc. of the supernatant broth was removed from one of these tubes and 1 cc. of 100 per cent sucrose added as before. The cells were allowed to remain in contact with the sugar for one-half hour at room temperature. The other tube of centrifuged cells was used as a broth control. In this case all but 2 cc. of the supernatant broth was removed and the cells resuspended in the 2 cc. volume of broth. After removing

Table 41. The thermal resistance of E. coli 52 exposed to 50 per cent sucrose 30 minutes, then washed in 0.85 per cent NaCl.

Treatment of cells	Agar	Count per cc. Before heating	After heating, 54.5°C., 5 minutes.	Survival per million
Exposed to 50 per cent sucrose 30 minutes, washed 7 times and heated in 0.85 per cent saline.	Plain	280,000,000	340,000	1,200
	Dextrose	330,000,000	410,000	1,300
Exposed to 50 per cent sucrose 30 minutes, not washed, heated in the sucrose solution.	Plain	840,000,000	400,000,000	476,000
	Dextrose	900,000,000	400,000,000	444,000
Centrifuged from broth, resuspended and heated in broth.	Plain	1,000,000,000	1,300,000	1,300
	Dextrose	960,000,000	4,200,000	4,400

Table 42. The thermal resistance of E. coli 57 exposed to 50 per cent sucrose, then washed in 0.85 per cent NaCl.

Treatment of cells	Count per cc.		
	Before heating	After heating 54.5°C., 5 minutes	Survival per million
Exposed to 50 per cent sucrose 1 hour, washed 7 times in 0.85 per cent saline and heated in saline.	15,000,000*	740,000	29,000
Exposed to 50 per cent sucrose 6 hours, not washed, and heated in the sugar suspension.	43,000,000	8,800,000	205,000
Centrifuged from broth resuspended and heated in broth.	1,100,000,000	150,000,000	14,000

* 1 per cent sucrose agar used in all plates.

1 cc. portions of these three suspensions for plating before heating, the tubes were placed in the water bath at 54.5°C. for 5 minutes. Plates before and after heating were made with plain and dextrose agars.

Table 42 shows the results of a similar experiment except that E. coli 57 was employed and the cells were exposed to the sucrose solutions for 1 hour. It is quite evident

from the data in tables 41 and 42 that the protective action which 50 per cent sugar afforded cells was readily removed by washing the cells in 0.85 per cent NaCl solution. The cells heated in saline and in broth suspensions were quite susceptible to heat, whereas the presence of sugar during heating resulted in an increased resistance. Attention is called to the data in table 42 which indicate that sucrose showed a definite protective effect on E. coli 57. Although sucrose usually has not shown any protective action for this strain of E. coli, such variations frequently have been observed with every culture employed in this study. The inconsistency of response to protective action, even though every effort has been made to standardize the conditions, is, in itself, a suggestion that the phenomenon is affected by delicately balanced forces.

Another experiment to determine the effect of washing cells after exposure to sugar is reported in table 43. An 18 hour culture of E. coli 52 was centrifuged, and the concentrated cell suspension was divided into two parts, one of which was exposed to 50 per cent sucrose for 1 hour. After the period of exposure, each cell suspension (with and without sugar) was divided into two parts, one of which was washed 5 times in 0.85 per cent NaCl. Thus there were available two tubes of cells which had been exposed to sugar,

Table 43. The effect of washing *E. coli* 52 on its resistance to heat (54.5°C., 5 minutes).

Treatment of cells		Agar	Count per cc. Before heating	Count per cc. After heating, 54.5°C., 5 minutes	Survival per million
Suspended in 50 per cent sucrose	Not washed	Plain	11,000,000	740,000	67,000
		Dextrose	80,000,000	8,000,000	100,000
	Washed in saline	Plain	15,000,000	60	4
		Dextrose	56,000,000	0	0
Suspended in broth	Not washed	Plain	400,000,000	14,000	35
		Dextrose	400,000,000	4,000	10
	Washed in saline	Plain	5,000,000	40	8
		Dextrose	10,000,000	0	0

one of which had been subsequently washed. These were plated before and after heating at 54.5°C. for 5 minutes, using plain and dextrose agars.

The results given in table 43 confirm the observations in previous experiments that washing of E. coli 52 removed any protective effect which sugar may have afforded the cells. The cells which had not come in contact with sugar were susceptible to heat, whereas those cells exposed to 50 per cent sucrose and not washed were protected to about the same degree as was observed in previous experiments.

The Protective Action of Hypertonic Solutions Against the Coagulation of Non-living Protein Systems.

It is obviously difficult to demonstrate changes in the physical status of the colloidal system of a minute bacterial cell except by indirect means. Several investigators have obtained convincing evidence of coagulation by observing the protoplasm of relatively large cells such as yeast under the ultramicroscope. With small bacterial cells, however, it is necessary to resort to various schemes which will reveal the changes in physical status of the cell by inference and analogy.

If the coagulation of cell colloids follows simple well defined laws of colloid chemistry, then the protective action afforded by sugars should be readily demonstrable with non-

living colloids. The following experiments were designed to investigate such a possible parallelism.

Effect of hypertonic dextrose and sucrose solutions on the coagulation of egg albumin. On a basis of the foregoing assumption it was reasoned that if the increased thermal resistance of cells heated in the presence of hypertonic sugar solutions is the result of delayed coagulation, it should be possible to demonstrate the same thing with coagulable proteins such as egg albumin.

By serial dilution of 8-molal sucrose and dextrose solutions, 6, 4, 3, 2, 1, 0.5, 0.2, and 0.1-molal concentrations of each sugar were prepared. From these a series of tubes were prepared each containing 1 cc. of one of the above concentrations of sugar. To each tube 1 cc. of fresh egg albumin was added and thoroughly mixed with the sugar solution, thus reducing the concentration of sugar to one-half its original concentration. Tubes containing a mixture of equal parts of water and egg albumin, and also tubes containing undiluted egg albumin were prepared as controls.

The entire series of tubes for both sugars were placed simultaneously in a water bath at the desired temperature and the time of coagulation noted. It was necessary to adopt an arbitrary standard of turbidity for coagulation in order that the readings could be rendered uniform for the various tubes in the series. The albumin was arbitrarily

Table 44. The effect of equimolal concentrations of dextrose and sucrose on the coagulation of egg albumin.

Molality of sugar	Minutes required to coagulate egg albumin at					
	60°C.		65°C.		100°C.	
	Dextrose	Sucrose	Dextrose	Sucrose	Dextrose	Sucrose
4	*	*	*	*	0.5	0.5
3	*	*	34	52	0.2	0.2
2	75	*	6	11.5	0.2	0.2
1	22	31	4	4.5	0.2	0.2
0.5	8	10	3	3.5	0.2	0.2
0.2	6	7	3	3.5	0.2	0.2
0.1	6	7	3	3.5	0.2	0.2
0.05	5	5	2	2	0.2	0.2
Water - egg 1:1	3	3	2	2	0.2	0.2
Undiluted egg	4	4	2	2	0.2	0.2

* Not coagulated after 24 hours.

regarded as coagulated when it was no longer possible to identify and differentiate the letters on a typewritten page held behind the tube; the size of the tubes was 10 x 100 mm.

The data in table 44 show the time required for coagulation of egg albumin at 60, 65, and 100°C. when mixed with equal parts of various equimolal concentrations of dextrose and of sucrose. At 60°C. a material increase in the time required for coagulation was observed when the sugars were present in 1-molal or greater concentrations. When 3-molal and 4-molal solutions of either sugar were employed coagulation was prevented for at least 24 hours. It is especially significant that sucrose afforded more protection than dextrose. The albumin coagulated in 75 minutes at 60°C. in the presence of 2-molal dextrose, whereas an equimolal solution of sucrose prevented coagulation for 24 hours.

When the experiment was repeated at 65 and 100°C. much more rapid rates of coagulation were observed. For example, with the 2-molal solutions the albumin failed to coagulate in 24 hours at 60°C., but when exposed to 65°C. coagulation occurred in 34 minutes in the dextrose and 62 minutes in the sucrose solutions. At 100°C. coagulation in all concentrations employed occurred in relatively few seconds.

Special attention is called to the observations that protective action is most effectively demonstrated when high concentrations of sugar are employed at relatively low tem-

peratures. The tremendous increase in protective action when the concentration of sugar is increased from 2- to 3-molal, together with the very marked decrease in time of coagulation when the temperature is elevated 5°C. is in harmony with the general observations made with cell suspensions in many of the preceding experiments. It was repeatedly demonstrated in preliminary experiments with cell suspensions that protective action was difficult to demonstrate at the temperature and time of exposure employed in pasteurization. The results reported in table 44 suggest that the rate of reaction in this case may be greatly affected by the temperature and that protective action may completely escape attention when higher temperatures are employed. This point is of practical significance in the possible application of the principle of protective action to the pasteurization of ice cream mix.

The effect of various solutes on the inactivation of rennin by heat. To a series of tubes each containing 2 cc. of rennet extract was added an equal volume of one of the following substances; conductivity water, 1-molal sodium chloride, 1-molal calcium chloride, 4-molal sucrose, and glycerol (sp. gr. 1.25). These, together with a control tube containing undiluted rennet were placed in a water bath at 70°C. At various time intervals 1 drop of the rennet mixture from each tube was removed and added to tubes contain-

ing 10 cc. of fresh, raw milk. The tubes of milk were held at room temperature and observed for the time of coagulation. Inactivation of the rennin should decrease its ability to induce coagulation of the milk. If the inactivation were incomplete and reversible, the rennin might be sufficiently peptized by the milk to induce coagulation, but only after a delay commensurate with the degree of injury.

Table 45. The effect of various substances on the inactivation of rennin by heat.

Minutes rennet mixture exposed to 70°C.	Coagulation time in minutes of 10 cc. of milk by 1 drop of rennet mixture.					
	Material added to rennet (equal volume).					
	0	Conductivity water	1-molal sodium chloride	1-molal calcium chloride	4-molal sucrose	glycerol
0	34	30	29	20	53	20
2.5	40	72	41	22	52	24
5.0	109	*	319	22	51	31
7.5	162		*	36	27	36
10.0	314			39	25	100
12.5	350			101	61	106
15.0	*			109	70	135
20.0				250	65	260
30.0				394	55	*
45.0				*	40	
60.0					38	
100.0					50	
120.0					50	
150.0					44	
180.0					70	
240.0					55	
300.0					60	

* not coagulated after 24 hours.

It will be observed from table 45 that 1 drop of the undiluted rennet before heating induced coagulation of 10 cc. of milk in 34 minutes. After heating 2.5, 5.0, 7.5, 10.0, and 12.5 minutes, the coagulation times increased to 40, 109, 162, 314, and 550 minutes respectively. After exposure to 70°C. for 15 minutes the rennin was so completely inactivated that it could not induce coagulation of the milk even after 24 hours. All tubes of milk not coagulated after 24 hours were still sweet to the taste.

The rennet extract which was diluted with water was completely inactivated after 5 minutes exposure. This is significant in that it agrees with the decreased thermal resistance observed with certain bacterial cells in water suspensions. Likewise, it will be observed in table 45 that 4-molal sucrose very effectively protected rennin against destruction by heat. Even after 5 hours exposure at 70°C. the rennin coagulated milk in 60 minutes. Glycerol and 1-molal calcium chloride also afforded definite protective action although not to the same degree as sucrose. Sodium chloride apparently hastened the inactivation of the rennin. Controls on this experiment were prepared by adding comparable quantities of water, sodium chloride, calcium chloride, sucrose, and glycerol to tubes of milk. One drop of the undiluted rennet extract heated for the indicated time intervals was added to the tubes. The data were not incorporated in the

table as the coagulation times were identical with those observed for the undiluted rennet except in the case of the milk to which calcium chloride was added. As might be expected the addition of calcium chloride to the milk reduced the time of coagulation a few minutes in each case.

These results further emphasize the significance of dehydration as a factor in the stabilization of a colloid to precipitation by heat. The observations are so completely in accord with those made with cell suspensions that the operation of the same set of factors is forcibly impressed.

DISCUSSION

General Statements

One of the salient features in the phenomenal development of microbiology has been the perfection of methods for the destruction of microorganisms without accurate knowledge of the fundamental mechanism of the death of the cell. Early recognition of the immediate need of practical information regarding disinfectants and disinfection processes has led to an empirical rather than a fundamental approach to such studies.

Thermal death point studies on numerous microorganisms and phenol coefficient determinations on many disinfectants and antiseptics have been the answer to an insistent demand for information on one of the most important aspects of microbial control, that is, cell destruction. As might be expected in any development under the pressure of necessity, the hastily prepared foundation soon proved inadequate for the superstructure. Many of our concepts of the thermal resistance of cells or the relative values of chemicals in cell destruction will have to be revised because of the false

basis upon which they have been founded. Much of the work on the resistance of cells must be repeated and reinterpreted in the light of newer concepts of the mechanism of cell destruction.

In measuring the resistance of a cell to heat or chemicals it is usually customary to determine the minimal exposure requisite for destruction. The inability of the cell to grow in or on the medium provided is accepted as satisfactory evidence that the cell is dead. There is a tendency to draw a sharp line of demarcation between viable and non-viable cells. That no such line exists is amply proved in the accompanying data as well as other data in which organisms have been subjected to minimal exposures of heat or chemicals.

The data in this paper lend support to the concept that the individual cell does not die suddenly after a given dosage of chemical or a certain exposure to heat. Unfavorable environmental factors induce changes in the cell which behave in accordance with well established principles of colloidal chemistry. It is believed that all of the salient features of the data presented can be explained on a basis of the theory of cell destruction outlined by Bancroft and Richter (1931). This concept permits of a logical basis for elucidating the protective action, increased growth in carbohydrate media, increased sensitivity of cells to water,

and other observations made in connection with this study.

Minimal Alterations in the Colloidal System of the Cell.

Stimulation. The stimulative action of minimal amounts of even the most toxic substances long has been a matter of record. At first stimulation seems incongruous with cell destruction, and it is perplexing to explain such opposite processes on a basis of a uniform mechanism. One of the appealing features of the theory outlined by Bancroft and Richter (1931) is that it enables one to harmonize many such apparently incompatible, yet well established, observations regarding the death of the cell.

If the vital processes of the cell are activated by shifts in the status of the equilibria between the various chemical reactions involved, it seems logical to assume that the selective adsorption of a specific substance might induce the displacement of some compound active in an equilibrium. The resulting increased concentration of the displaced compound in the continuous phase in turn may induce increased activity of metabolic processes. Obviously such displacement could effect stimulation only through a limited zone. One would expect the width of such a zone to vary with the selective adsorbing properties of different com-

pounds and to be so narrow as to be difficult to detect with highly toxic substances. The narrow zone of stimulation for some substances is so rarely observed and the chemicals are so universally regarded as poisons that it is surprising to learn of the existence of such stimulative action. Other compounds less actively adsorbed prove stimulative over such a wide range that it becomes equally surprising to find that they may be destructive. The experiments described in this paper were not designed to show stimulative action, but the point is discussed here because of its fundamental bearing on the concept of the stability of the cell.

Reversible coagulation and peptization. The degree of dispersion of the colloids of the normal cell presumably is optimum for maximum stability. At least the evidence afforded by observation of narcotized cells under the ultramicroscope indicates that agglomeration of the cell colloids is among the first manifestations of injury. (Hadson and Meisel (1926), Hirschfelder and Decherd (1927), and others). The reversibility of incipient coagulation of proteins is readily demonstrable either in vitro or in vivo. As the agglomeration of the colloids becomes progressively more pronounced, the cell becomes more sluggish and eventually dormant. Such a cell is narcotized and may be revived if placed under conditions conducive to peptization of the col-

loids to their normal degree of dispersion.

It is apparent that the more advanced the degree of coagulation, the greater will be the difficulty encountered in peptization. Similarly, one should expect considerable differences in the peptizing qualities of various media. The coagulated colloids of an injured cell may be peptized by one medium and not by another. Obviously reversibility of coagulation is a purely relative matter and depends upon a complementary relationship with the peptizing qualities of the medium employed. Any determination of minimum lethal exposure is therefore subject to criticism on a basis of the uncertainty of the death of the organism.

The data presented in many of the tables show that when heated cells are plated on plain agar and on various carbohydrate agars, the number capable of regeneration is usually larger in the latter media. In any population of mildly injured cells, varying degrees of coagulation can be expected. The less resistant forms will have been coagulated to such an advanced degree that even the best medium employed will not induce regeneration. It would involve conjecture, however, to predict, on the basis of such limited information, that coagulation had gone so far that no medium or animal inoculation could ever induce peptization. Nevertheless, that is essentially the inadequate basis of any pronouncement that a cell has been killed. Obviously, when very

extreme exposures have been employed, death may be considered fairly certain, but when minimal exposures have been employed the conjecture involved discourages the identification of a "death point".

The most resistant cells in a population may be so slightly coagulated that even the poorest medium may induce a return to normal dispersion of the cell colloids. A survey of the comparative counts on plain and carbohydrate agars shows that occasionally the latter offered very little advantage, especially if the injury was either very severe or very slight. In either of these cases one would expect little difference in the peptizing qualities of the two media.

The number of survivors capable of withstanding a given treatment is governed by the extent to which the medium employed can effpotively reverse the coagulation processes induced in the individual cells concerned. The extent of reversibility in turn will depend, (1) upon the severity of the exposure, (2) the distribution of resistant and non-resistant types in the cell population, (3) the presence of protective agencies, and (4) the peptising qualities of the medium.

Radical Alterations in the Colloidal System of the Cell.

Irreversible coagulation. When the cell colloids have agglomerated to the extent that all efforts fail to peptize them, the cell may be regarded, with reservations, as irreversibly coagulated or dead. The impracticability of proving such a point beyond all peradventure of doubt is illustrated by the reports of extreme delay of several hundred days in the germination of heated spores, (Esty and Meyer 1922) and of the revival of anthrax spores after 40 days exposure to $HgCl_2$ (Süpfle and Müller 1920).

Failure to recognize the limitations imposed by experimental procedure is the basis of criticism of most experimental work dealing with cell resistance. It is quite proper to conclude that the colloids of a cell are irreversibly coagulated with respect to a given medium, but obviously the conclusion should be confined to the observation, especially if minimal exposures have been employed. In many of the experiments reported in this paper, for example, it has been shown that the presence of sugar retards the coagulation of cells by heat. Cells heated in water, on the other hand, were incapable of growing on the media supplied. Attempts to revive these injured cells by adding

the sugar after heating (tables 35 and 36) met with failure. That the injury was irreparable and the coagulation irreversible was only partly demonstrated.

Factors Affecting Protective Action.

Adsorption. A survey of the experimental results in this paper leaveslittle doubt that hypertonic solutionsof dextrose and sucrose afford protection to certain cells against the destructive action of heat. The possible role which adsorption may play in this connection is suggested by the fact that the protective principle may be washed from the cell (tables 41-42-43).

Permeability. The importance of permeability in protective action is suggested by the results of many experiments. Prolonged exposure of cells to hypertonic solutions in milk (table 6) and in broth, (tables 8, 11, 12, 14, 15, and 16) results in an actual increase in the number of cells capable of withstanding a given heat treatment, although the total number of cells in the unheated samples shows a marked decline. It is believed that this is explicable on a basis of different degrees of permeability of the cells in the population. There are apparently two opposite results from the same force. The high osmotic pressure induces the transfer of water across the cell wall which eventually leads

to death of the cell. If the process is sufficiently slow there is a period in which the withdrawal of water up to a certain point increases the permeability of the cell for the solute responsible for hypertonicity. The prolonged action at lower temperatures of equimolal solutions of dextrose and sucrose in water failed to show the increase in numbers of cells that was observed in the broth and milk suspensions.

The comparison of the protective action of various sugars in 50 per cent solutions (table 10) and of equimolal concentrations of dextrose and sucrose (tables 19, 20, 21, 22, 23, and 24) suggest very strongly that penetration of the cell wall must play an important role in the regulation of protective action. Variations in the selective permeability of organisms may result in one sugar affording greater protective action than another for a given species, whereas the relative protective qualities of the sugars may be the reverse for some other organism (table 34).

Cells grown in dextrose broth were found to be somewhat more effectively protected by sucrose than cells grown in plain broth (tables 35 and 36). However, growth in dextrose broth did not seem to afford much advantage to cells heated in dextrose (tables 37).

Perhaps the most convincing evidence that the permeability of the cell membrane is an important factor in pro-

tective action is that the cells washed in water were rendered more sensitive to sugar (table 40), whereas cells washed in saline were not so affected (table 39).

Osmosis. If E. coli 52 is heated in a series of solutions of dextrose with increasing osmotic pressures, there is an unmistakable parallel increase in the protective action. A similar series of equimolal concentrations of sucrose will show considerably greater protective action than the dextrose solutions, (tables 19, 20, 21, 22, 23, 24). This suggests that although osmotic pressure is an important factor in the protective action afforded by sugars it is not the only agency involved. If the protective action is to be explained on a basis of the transfer of water, obviously osmotic pressure would play an important part. On the other hand, the effective osmotic pressure at the cell surface is regulated not only by the molecules in solution but by the relative permeability at each individual cell surface. If variation in the permeability of individual cells in not recognized, then cells suspended in hypertonic solutions would suffer alike and simultaneously. The results in this paper show quite definitely that uniformity in response is not to be expected.

Temperature. The experiments in which cells were subjected to hypertonic solutions at various sub-lethal temperatures (tables 28, 29, 30, 31, 32, and 33) and then heat-

ed, brought out some rather significant points. The death rates in the unheated samples were more rapid in dextrose in each case than in sucrose, and in the aqueous suspensions growth usually occurred. The more rapid disappearance of cells in the dextrose than in the sucrose suspensions is in harmony with the observations made at higher temperatures in other experiments. This further suggests that any protective action which these sugars may afford the cell must be the result of incipient coagulation, a process which ultimately leads to the destruction of the cell.

Sensitivity to water. The rather sudden change in the behavior of cells in water at 54.5°C. and at 44.5°C., as well as at lower temperatures, deserves special note. It was observed in many of the experiments that heating E. coli 52 in water to 54.5°C. resulted in almost complete destruction, whereas similar exposure in hypertonic solutions was less destructive. When the temperature of exposure was lowered to 44.5°C. an entirely different picture was presented; the hypertonic solutions exhibited their destructive action at a slower rate, but the cells in water were not destroyed. (figure 9). Exposures at still lower temperatures likewise showed the same tendency to kill the cells at progressively lower rates with lower temperatures of exposure, whereas the

aqueous suspensions showed growth. This suggests that as the temperature is increased from 44.5 to 54.5°C. there is a rapid increase in the sensitivity of E. coli 52 to heat in water, and that the protective action of sugars may be traceable in part to lowering the degree of sensitivity. The rate of destructive action of dextrose and sucrose likewise increases in the temperature range between 44.5 and 54.5°C., but not to the same extent as in the aqueous suspensions with the same temperature increment.

The protective action of hypertonic sugar solutions against the coagulation of egg albumin and the destruction of rennin further suggests that the protection is afforded by retarding the rapid dehydration and coagulation of the colloidal complex. The addition of water hastened the time of coagulation or destruction of the enzyme (tables 44 and 45), and the addition of hypertonic sugar solutions retarded the same process. The parallelism between the observations with non-living proteins and those made with living cells is quite apparent.

Mechanism of Protective Action

Although any explanation of the mechanism of protective action can be based only on indirect evidence, it should be made to harmonize with the following rather unrelated points:

(1) Not all cells are protected by sugars; some are protected by one sugar, some by another; (2) Permeability apparently plays an important part in regulating the protective action of individual cells as well as regulating the resistance of strains or species; (3) Protective action is directly related to osmotic pressure values for an individual compound, but the relationship does not apply for different compounds; (4) Prolonged contact of the cells in hypertonic solutions in water shows no increase in protective action whereas cells suspended in hypertonic broth or milk give evidence of an increase in the number of cells capable of withstanding a given heat treatment.

Interpreting these observations in the light of the evidence available from other workers it is believed that protective action is a simple manifestation of well established principles. When certain cells are suspended in water and heated to gradually increasing temperatures, within a very narrow temperature gradient the rate of death greatly increases. Comparable cells suspended in hypertonic dextrose solution show a more uniform increase in death rate through the various temperature ranges. In sucrose solution there is a still lower rate of death at parallel temperatures, and hence sucrose manifests the greater protective influence.

Death in the water suspension is due to a sensitivity to water, the penetration of which is greatly accelerated by

heat. If the stabilizing charge of the cell colloid is fixed within relatively narrow limits, it is reasonable to suspect that the increased mobility of ions resulting from an excess of water might alter the charge beyond the limits of stability.

Death in the sugar suspensions is apparently due to a different set of factors. The principal process involved, desiccation, is immediately destructive to many of the cells in the population. Like other destructive agencies, however, the incipient changes induced may be quite in contrast to destruction. The cells which more slowly reach an equilibrium with the hypertonic solution exhibit a higher degree of thermal resistance. The presence of some protein such as milk or broth is believed to alter the conditions at the surface in such a manner as to slow down the rate of adjustment sufficiently to cause an actual increase in the number of thermal resistant cells. In other words, it is believed that the so-called protective action of sugars for certain cells is due to imposing another destructive agency which has a lower temperature quotient than the same cells would experience in water.

Limitations of the Work

Obviously the ultimate solution to a problem of such wide scope would involve an extensive survey of the tendency

of various organisms to exhibit this phenomenon. The possibility that pathogenic organisms may be so affected remains unexplored. The practical aspects of the problem and their application to the preservation of sweetened condensed milk, sorghum, honey, fruits canned in heavy syrup, and the pasteurization of ice cream mix have not been attempted. More study should be given to the effect of varying the permeability of the cell on the comparative rates of death in water and in sugar suspensions. This obviously involves a study of ion effects on permeability.

CONCLUSIONS

1. The addition of sugar to ice cream mix may increase the thermal resistance of the microflora.

2. Hypertonic solutions of dextrose and sucrose made up in broth, milk, water or ice cream mix afford certain cells definitely greater protection against heat than when heated in water suspensions.

3. The addition of sugar after heating does not induce an increased survival of the cells employed in this study.

4. The variation in the peptizing qualities of different media for the cells which have been only reversibly coagulated by minimal exposures, suggests caution in all studies based upon the death of the cell as measured by cultural methods.

SUMMARY

This work involves a study of the increased resistance manifested by certain microorganisms when heated in the presence of high concentrations of sugars. The significance of this phenomenon in the pasteurization of ice cream mix is demonstrated and the probable relation to the preservation of condensed milk, canned fruits, honey, sorghum, etc. is suggested.

Within the limitations imposed by the experimental procedure it has been possible to demonstrate some of the fundamental points having a bearing on the mechanism of this protective action. The permeability of the individual cell apparently plays an important part in regulating the rate of death in the water and in sugar suspensions.

The protective action increased with increased osmotic pressures in a series of concentrations of a given sugar, but equimolal solutions of the different sugars did not show the same protective action. Maltose and lactose gave little or no protection to the cells studied.

Prolonged exposures of certain cells to hypertonic solutions in broth and milk resulted in an actual increase in the

numbers of cells capable of withstanding a given heat treatment. Prolonged exposures in aqueous hypertonic solutions showed a decline in the thermal resistance of the survivors.

Not all cells exhibit the phenomenon of increased thermal resistance in hypertonic solutions. It is believed to be limited to those cells which are highly sensitive to water at slightly increased temperatures. The rapid death rate in water is replaced by a slower death rate in the hypertonic solution at the same temperature. Washing of cells in water or broth renders them more susceptible to the destructive action of hypertonic solutions. Washing of cells after exposure to hypertonic solutions removes any protective action which the sugar solution affords the cells in the unwashed portion.

Parallel with the protective action for cells, hypertonic sugar solutions have been shown to delay or even prevent the coagulation of egg albumin and to retard the inactivation of rennin by heat.

LITERATURE CITED

Adametz, L., 1889. Saccharomyces lactis eine neue Milchzucker vergärende Hefeart. Centbl. Bakt., II Abt., 5:114-120.

Anzulovic, J. V., 1932. A study of Escherichia coli in ice cream. Jour. Bact., 23:56-57.

Ayers, S. H., and Johnson, W. T., jr., 1914. Ability of streptococci to survive pasteurization. Jour. Agr. Res., 2:321-330.

Ballantyne, T. N., 1930. On certain factors influencing the survival of bacteria in water and in saline solutions. Jour. Bact., 19:303-320.

Bancroft, W. D., 1913. The theory of emulsification. Jour. Phys. Chem., 17:501-519.

_____, and Richter, G. H., 1931. The chemistry of disinfection. Jour. Phys. Chem., 35:511-530.

_____, and Rutzler, J. E., jr., 1931. The denaturation of albumin. Jour. Phys. Chem., 35:144-161.

Barthel, Chr., and Stenström, O., 1912. Untersuchungen über die Widerstandskraft der Tuberkelbazillen gegen Erhitzung in Milch. Ztschr. f. Fleisch-u. Milchhyg., 22:137-148, and 173-187.

Bartlett, C. J., and Kinne, F. B., 1913. Resistance of microorganisms suspended in glycerine or oil to the sterilizing action of heat. Science, n. s., 38:372.

Beavens, E. A., 1930. The effect of lactose on the survival of Escherichia coli when heated to 145°F. for thirty minutes. Jour. Dairy Sci., 13:91-92.

Bechhold, H., 1909 a. Desinfektion und Kolloidchemie. Ztschr. f. Chemie u. Indus. Kolloide, 3:22-25.

———————, 1909 b. Halbspezifische chemische Desinfektionsmittel. Ztschr. f. [illegible] Hyg. u. Infektionskrank., 64:115-142.

———————, 1919. Colloids in biology and medicine. Trans. by J.G.M. Bullowa. 464 p. D. Van Nostrand, New York.

———————, and Reiner, R., 1923. Adsorptivdesinfektion in Gegenwart anderer Adsorbentien. Ztschr. f. Hyg. u. Infektionskrank., 96:17-84.

Beck, W. A., 1931. Variations in the Og of plant tissues. Plant Physiol., 6:515-525.

Bellinsson, A., 1929. Thermostabilisation der Eiweisslösungen mit Rohrzucker und Glycerin. Biochem. Ztschr., 213:399-405.

Beutner, R., 1933. Physical chemistry of living tissues and life processes. 577 p. Williams and Wilkins, Baltimore.

Breinl, F., 1920. Über das Verhalten grampositiver und gramnegativer Bakterien zu den Halogenen. Ztschr. Immun. Forsch., Orig., 29:343-348.

Brooks, M. M., 1918. Comparative studies on respiration III. The effect of ether on the respiration and growth of Bacillus subtilis. Jour. Gen. Physiol., 1:195-201.

———————, 1920-1921. Comparative studies on respiration XIV. Antagonistic action of lanthanum as related to respiration. Jour. Gen. Physiol., 3: 337-342.

Brooks, S. C., 1918. A theory of the mechanism of disinfection, hemolysis, and similar processes. Jour. Gen. Physiol., 1:61-80.

Brown, C. W., and Peiser, K., 1916. Keeping qualities of butter V. Pasteurization and its influence. A study of the factors which influence the resistance of lactic acid bacteria to heat. Mich. Agr. Expt. Sta., Tech. Bul. 30, 18 p.

Browning, C. H., and Gilmour, W., 1913. Bacterieidal action and chemical constitution with special reference to basic benzol derivatives. Jour. Path. and Bact., 18:144-146.

Brundy, V., 1908. Über die Beziehung zwischen der Farbbarkeit der Bakterien nach Gram und ihrer Permeabilität. Centbl. Bakt., II Abt., 21:62-80.

Buchanan, R. E., and Fulmer, E. I., 1928-1930. Physiology and biochemistry of bacteria, Vol. I, 516 p. Vol. II, 709 p. Vol. III, 575 p., Williams and Wilkins, Baltimore.

Bullock, H., 1913. The resistance of spores to heating in anhydrous fluids as glycerine and similar substances. Jour. Hyg., (London), 13:168-177.

Burke, G. S., 1919. The effect of heat on the spores of Bacillus botulinus: Its bearing on home canning methods. Part I. Jour. Amer. Med. Assoc., 72: 88-92.

Butterfield, C. T., 1932. The selection of dilution water for bacteriological examination. Jour. Bact., 23: 355-368.

Chick, H., 1910. The heat coagulation of proteins. Jour. Physiol., 40:404-430.

Clowes, G. H. A., 1916 a. Protoplasmic equilibrium I. Action of antagonistic electrolytes on emulsions and living cells. Jour. Phys. Chem., 20:407-451.

_____, 1916 b. Antagonistic electrolyte effects in physical and biological systems. Science, n. s., 43:750-757.

_____, 1918. On the action exerted by antagonistic electrolytes on the electrical resistance and permeability of emulsion membranes. (preliminary note). Soc. Expt. Biol. and Med., Proc., 15:108-111.

Cochran, C. B., and Perkins, J. H., 1914. The effect of high temperature on yeast. Jour. Ind. and Engin. Chem., 6:480.

Committee on Dairy Products and Eggs, American Public Health Association, Report, 1930. Amer. Jour. Pub. Health, 20:492-494.

Cook, W. D., 1931. Studies on the sterilization of solutions of glucose and sucrose. Doctoral thesis, Library, Iowa State College, Ames, Iowa.

Cooper, E. A., 1912. On the relations of phenol and meta-cresol to proteins. A contribution to our knowledge of the mechanism of disinfection. Biochem. Jour., 6:362-387.

_____, and Mason, J., 1926. A contribution to our knowledge of disinfectant action II. The relation of phenols and amines to proteins. Jour. Phys. Chem., 32:868-875.

_____, and Sanders, E., 1927. On the relations of phenol to proteins and other colloids. A contribution to our knowledge of disinfectant action. Jour. Phys. Chem., 31:1-22.

Cramer, E., 1891. Die Ursache der Resistenz der Sporen gegen trockne Hitze. Arch. Hyg., 13:71-113.

Daniels, J. F., 1908-1909. The adjustment of Paramecia to distilled water. Amer. Jour. Physiol., 23:48-63.

Davenport, C. B., and Castle, W. E., 1895. Acclimatization of organisms to high temperatures. Arch. f. Entwickl. Mech. Organ., 2:227-249.

Dickson, E. C., Burke, G. S., Beck, D., Johnson, J., and King, E., 1928. Studies on thermal death time of spores of Clostridium botulinum. Jour. Amer. Med. Assoc., 79:1239-1240.

_____, _____, and Ward, E. S., 1919. Botulism. A study of the resistance of the spores of Bacillus botulinus to various sterilizing agencies which are commonly employed in the canning of fruits and vegetables. Arch. Int. Med., 24:551-599.

Dolloff, A. F., 1926. A simple synthetic medium for the cultivation of bacteria. Science, n. s., 64:254.

Dougherty, M. W., 1930. Studies on thermal death points of yeasts. Thesis, Library, Univ. of Ill., Urbana, Ill.

Dreyer, G. C., and Walker, E. W. A., 1912-1913. Resistance of microorganisms suspended in glycerine or oil to the sterilizing action of heat. Jour. Path. and Bact., 17:142-148.

Dubos, R., 1930. The bacteriostatic action of certain components of commercial peptones as affected by conditions of oxidation and reduction. Jour. Expt. Med., 52:331-345.

Kijkman, C., 1918. Expériences osmotiques avec des spores bactéries. Arch. Néerland Physiol., 2:618-620. (Cited by Buchanan and Fulmer, 1930, Vol. II, p. 198). Original not seen.

Eisenberg, P., 1910. Ueber die Tuschedifferenzierung gramnegativer Bakterien. Centbl. Bakt., I Abt., 56: 183-186.

_____, 1918. Ueber spezifische Desinfektion. Der Militärarzt S. 577, (Cited in Centbl. Bakt., I Abt., Ref., 66:430-431). Original not seen.

_____, 1918. Untersuchungen über spezifische Desinfektionsvorgänge III Mitteilung: Ueber die Wirkung von Salzen und Ionen auf Bakterien. Centbl. Bakt., I Abt., Orig., 82:69-207.

_____, and Okolska, M., 1913. Untersuchungen zur Theorie der Desinfektion. Centbl. Bakt., I Abt., Orig., 69:312-346.

Englehart, N., 1922. Untersuchungen über den Mechanismus der Sublimatwirkung auf Bakterien. Desinfektion., S. 63 u. 81. (Cited in Centbl. Bakt., I Abt., Ref., 75:316-317). Original not seen.

v. Esmarch, E., 1889. Die Milzbrandsporen als Testobject bei Prüfung von Desinficienten. Ztschr. f. Hyg., u. Infektionskrank., 5:67-72.

Esty, J. R., 1920. The biology of Clostridium Welchii. Jour. Bact., 5:375-429.

_____, and Meyer, K. F., 1922. The heat resistance of spores of B. botulinus and allied anaerobes XI. Jour. Infect. Diseases, 31:650-665.

Fabian, F. W., and Coulter, E. W., 1930. Significance of colon-aerogenes group in ice cream I. Survival of Escherichia-Aerobacter group to pasteurization temperatures in ice cream. Jour. Dairy Sci., 13: 273-287.

Fabricius, N. H., and Hammer, B. W., 1931. Observations on the counting of bacteria in ice cream by the plate method. Ia. Agr. Expt. Sta., Bul., 285, p. 331-342.

Falk, I. S., 1923. The role of certain ions in bacterial physiology. A review. (Studies on salt action VII). Abstracts Bact., 7:35-50; 87-105; 133-147.

Fay, A. C., 1927. Thermo-tolerant organisms as a cause of so-called pin-point colonies. Jour. Bact., 13:347-377.

Ficker, M., 1898. Ueber Lebensdauer und Absterben von Pathogenen Keimen. Ztschr. f. Hyg., u. Infektionskrank., 29:1-74.

_____, 1908. Über die Resistenz von Bakterien gegenüber dem Trocknen. Ztschr. f. Hyg. u. Infektionskrank., 59:267-278.

Frank, B., 1888. Untersuchungen über die Ernährung der Pflanzen mit Stickstoff und über den Kreislauf desselben in der Landwirtschaft. Landw. Jahrb., 17:421-555. (Cited by Hibbard).

_____, 1891. Über das Verhalten der Lupinenkeimlinge gegen destilliertes Wasser. Landw. Jahrb., 20:233. (Cited by Hibbard).

Free, E. E., 1918. A colloidal hypothesis of protoplasmic permeability. Plant World, 21:141-150.

Freundlich, H., and Rona, P., 1917. Über die Sensibilisierung der Ausflockung von Suspensionskolloiden durch capillaraktive Nichtelektrolyte. Biochem. Ztschr., 81:87-106.

Fulmer, E. I., and Buchanan, R. E., 1923. Studies on toxicity. Jour. Gen. Physiol., 6:77-89.

_____, Nelson, V. E., and Sherwood, F. F., 1921. The nutritional requirements of yeast II. The effect of the composition of the medium on the growth of yeast. Jour. Amer. Chem. Soc., 43:191-199.

Gainey, P. L., 1912. The effect of toluol and CS_2 upon the micro-flora and fauna of the soil. 22nd Ann. Rept., Missouri Botanical Garden, St. Louis, 147-169.

Gegenbauer, V., 1921. Studien über die Desinfektionswirkung des Sublimats. Arch. Hyg., 90:23-81.

_____, 1922. Studien über Desinfektionswirkung wässeriger Formaldehydlösungen. Arch. Hyg., 90:239-253.

Gorini, C., 1915. Resistance of non-sporing bacteria in milk to the action of heat. Rend. R. Institut. Lombardo Sci. Lettere, 48:956-961. (Abstract in Analyst 41: 331). Original not seen.

_____, 1921. Further observations on the biology of the lactic acid bacteria. Centbl. Bakt., II Abt., 53:264-267.

Gössl, J., 1913. Zur Kenntnis der lipoidlösenden Desinfektionsmittel. Zur Theorie der Desinfektion II. (mitgeteilt von R. O. Herzog). Hoppe-Seyler's Ztschr. Physiol. Chem., 88:103-108.

Gruber, M., 1891. Über die Methoden der Prüfung von Desinfektionsmitteln. VII internationaler Kongress für Hygiene und Demographie zu London. Centbl. Bakt., I Abt., 11:115-117.

Gutstein, M., 1926. Das Ektoplasma der Bakterien. 5 Mitteilung: Farberischer Nachweis und chemischer Bau des Ektoplasmas der gramnegativen Bakterien. Centbl. Bakt., I Abt., Orig., 100:1-9.

_____, 1927. Zur Theorie der Desinfektion. Mikrochemische Untersuchungen über die Wirkung von Schwermetallen und Farbstoffen auf lebende Bakterien. Centbl. Bakt., I Abt., Orig., 104-410-429.

Hall, L. T., 1928. The theory of disinfection. Military Surgeon, 50:295-305.

Hansen, T., 1921. Oberflächenspannung und die antiseptische Wirkung von Desinfektionsmitteln. Hosp.-Tid., 64: 657,673. (Abstract in Centbl. Bakt., I Abt., Ref., 75:137-138). Original not seen.

Herzog, R. O., and Betzel, R., 1910. Zur Theorie der Desinfektion. Vorläufige Mitteilung. Hoppe-Seyler's Ztschr. Physiol. Chem., 67:311-313.

_____, and _____, 1912. Zur Theorie der Desinfektion. Hoppe-Seyler's Ztschr. Physiol. Chem., 74:221-241.

Hewlett, R., and Hall, G. T., 1911. The influence of the culture medium on the germination of anthrax spores with special reference to disinfection experiments. Jour. Hyg., (London), 11:473-480.

Hibbard, R. P., 1915. The question of toxicity of distilled water. Amer. Jour. Bot., 2:389-401.

Hirschfelder, A. D., and Dechert, C. M., 1927. Inhibiting effects of lipoids upon actions of antiseptics. Soc. Expt. Biol. and Med., Proc., 25:834-836.

_____, and Wright, H. N., 1930. Studies on the colloid chemistry of antisepsis and chemotherapy III. The ultramicroscopic examination of neoarsphenamine and of certain antiseptics, and their effects upon protein solutions. Jour. Pharmacol. and Expt. Ther., 39:13-37.

Höber, R., 1924. Physikalische Chemie der Zelle und der Gewebe, (fünfte Auflage), 608 p., Verlag von Wilhelm Englemann, Leipzig.

Höfler, K., 1926. Ueber die Zuckerpermeabilität plasmolysierter Protoplaste. Ztschr. Wiss. Biol., Abt. E, Plante, 2:454-475.

Holman, W. L., 1914. The relative longevity of different streptococci and possible errors in the isolation and differentiation of streptococci. Jour. Infect. Diseases, 15:293-308.

Horst, H., 1931. Zur Theorie der halbspezifischen Desinfektionswirkung. Centbl. Bakt., I Abt., Orig., 122:54-56.

Hotchkiss, M., 1925. Studies on salt action VI. The stimulating and inhibitive effect of certain cations upon bacterial growth. Jour. Bact., 8:141-162.

Isaacs, M. L., 1930. Factors which influence tests of bacterial survival I. The effects of varying periods of incubation of the survival test culture. Jour. Bact., 20:181-194.

_____, 1932. A theory of disinfection. Science, n. s., 75:46-48.

James, L. H., 1930. A sugar-tolerant member of the colon-aerogenes group. Jour. Bact., 19:145-148.

Jezler, A., 1926. Versuche über Jodbildung. II Mitteilung. Jodadsorption und ihre Beeinflussung durch das Ionenmilieu. Ztschr. Gesam. Expt. Med., 50:714-727.

Keho, H., 1921. Ein Beitrag zur Permeabilität des Pflanzenplasmas für die Neutralsalz IV. Biochem. Ztschr., 123:284-303.

Knaysi, G., 1930. The cell structure and cell division of Bacillus subtilis. Jour. Bact., 19:113-115.

_____, and Gordon, M., 1930. Disinfection III. Taking up of iodine by yeast cells. Jour. Infect. Diseases, 47:318-321.

Küster, E., and Rothaub. 1913. Verlauf des Adsorptionsprozessen bei der Einwirkung des Phenols auf Bakterien. Ztschr. Hyg. u. Infektionskrank., 75:205-223.

Lange, B., 1922. Keimmenge und Desinfektionserfolg. Ztschr. Hyg. u. Infektionskrank., 96:92-117.

Lepeschkin, W. W., 1911. Zur Kenntnis der chemischen Zusammensetzung der Plasmamembran. Ber. Deut. Bot. Gesell., 29A:724-726.

_____, 1926. Ueber physikalisch-chemische Ursachen des Todes. Biol. Zentbl., 46:480-482.

Leuch, G., 1906. Sind bei der bakteriziden Wirkung des Blutserums osmotische Vorgänge im Spiele? Arch. Hyg., 54:398-412.

Lewith, S., 1890. Ueber die Ursache der Widerstandsfähigkeit der Sporen gegen hohe Temperaturen. Arch. Expt. Path. u. Pharmakol., 26:341-354.

Liesegang, W., 1930. Die Bekämpfung von Rauch, Staub und Abgasen als hygienische Aufgabe. Ztschr. Desinfekt. u. Gesundhw., 22:331-338. (Cited by Bancroft and Richter).

Lloyd, F. E., 1915. The behavior of protoplasm as a colloidal complex. Carnegie Inst. Wash., 14:66-69.

Lochhead, A. G., and McMaster, N. E., 1931. Yeast infection of normal honey and its relation to fermentation. Sci. Agr., 11:351-360.

Locksmen, G., and Pilcher, H., 1928 a. Adsorption und Desinfektion: Versuche mit Quecksilber-und Silbersalzen. Ztschr. Hyg. u. Infektionskrank., 108: 135-145.

_____, and _____, 1928 b. Adsorption und Desinfektion; weitere Versuche über den Einfluss der Adsorption des Desinfiziens und der Keime auf den Desinfektionsverlauf. Ztschr. Hyg. u. Infektionskrank., 108:746-772.

Lode, G., 1929. Untersuchungen über die Ursachen des schwächenden Einflusses von Glyzerin auf die Desinfektionskraft von Desinfektionsmitteln. Centbl. Bakt., I Abt., Orig., 112:401-411.

Loeb, J., 1908. Studies on the physiological effect of the valency and possibly the electrical charges of ions I. The toxic and antitoxic effects of ions as a function of their valency and possibly their electrical charge. Amer. Jour. Physiol., 6:411-433.

_____, 1912. The apparent antagonism between electrolytes and non-conductors. Science, n. s., 35:111-112.

_____, 1913. Avogadro's law and the adsorption of water by animal tissues in crystalloid and colloid solutions. Science, n. s., 37:427-429.

_____, and Wasteneys, H., 1913. On the adaptation of fish (Fundulus) to higher temperatures. Jour. Expt. Zool., 12:543-557.

Loughlin, W. J., 1932. The denaturation of proteins IX. The effect of the addition of electrolytes on the viscosity of solutions of crystalline egg-albumin. Biochem. Jour., 26:1557-1565.

Lucké, B., and McCutcheon, M., 1932. The living cell as an osmotic system and its permeability to water. Physiol. Rev., 12:68-139.

McCulloch, E. C., 1932 a. The role of disinfection in the control of Bang's disease. Vet. Med., 27:456-461.

_____, 1932 b. Disinfection. Vet. Med., 27:505-510.

_____, 1933. The germicidal efficiency of chlorine and chlorine compounds. Vet. Med.,28:92-97.

Mac Dougal, D. T., 1923. Permeability and the increase of volume contents of living and artificial cells. Amer. Phil. Soc., Proc., 62:1-35.

Magoon, C. A., 1928. Studies upon bacterial spores I. Thermal resistance as affected by age and environment. Jour. Bact., 11:353-383.

Mathews, A. P., 1904. The nature of chemical and electrical stimulation I. The physiological action of an ion depends upon its electrical state and its electrical stability. Amer. Jour. Physiol., 11:455-496.

Meyer, A. G., 1917. Is death from high temperatures due to accumulation of acid in the tissues? Amer. Jour. Physiol., 44:581-585.

Meyerhof, O., 1918. Notiz über Eiweisszfällungen Narkotica. Biochem. Ztschr., 86:325-328.

Miller, E. C., 1931. Plant physiology. 900 p. McGraw Hill, New York.

Morrison, E. W., and Rettger, L. F., 1930 a. Bacterial spores I. A study of heat resistance and dormancy. Jour. Bact., 20:299-311.

_____, and _____, 1930 b. Bacterial spores II. A study of bacterial spore germination in relation to environment. Jour. Bact., 20:313-342.

Mudge, C. S., and Lawler, B. M., 1928. The effect of distilled water upon the tendency to colony formation upon petri plates. Jour. Dairy Sci., 11:436-445.

Murray, T. J., and Headlee, M. R., 1931. Thermal death point I. Spores of Clostridium tetani. Jour. Infect. Diseases, 48:436-450.

Nadson, G. A., and Meisel, M. N., 1926. Le mécanisme de l'action du chloroforme sur la matière vivante. Compt. Rend. Acad. Sci., (Paris), 183:82-83.

Nechkovitch, M., 1927. Rôle de la glycose dans le maintien de l'état normal des colloides in vivo. Arch. Internatl. Physiol., 28:285-292.

Nernst, W., 1890. Ein osmotischer Versuch. Ztschr. Phys. Chem., 6:37-40.

Neuberg, C., 1916. Hydrotropische Erscheinungen. I Mitteilung. Biochem. Ztschr., 75:107-176.

Noguchi, H., 1905. A study of the protective action of snake venom upon blood corpuscles. Jour. Expt. Med., 7:191-233.

Oldenbusch, C., Frobrisher, M., and Shrader, J. H., 1930. Thermal death points of pathogenic bacteria in cream and ice cream. Amer. Jour. Pub. Health, 20: 615-618.

Osterhout, W. J. V., 1911. The permeability of living cells to salts in pure and balanced solutions. Science, n. s., 34:187-189.

_____, 1913. Effect of anaesthetics on permeability. Science, n. s., 35:113-115.

_____, 1914. The effect of alkali on permeability. Jour. Biol. Chem., 19:355-343.

_____, 1915 a. Extreme alterations of permeability without injury. Bot. Gaz., 59:243-253.

_____, 1915 b. On the decrease in permeability due to certain bivalent kations. Bot. Gaz., 59:517-530.

_____, 1915 c. The effects of some trivalent and tetravalent kations on permeability. Bot. Gaz., 59:464-473.

_____, 1915 d. Normal and abnormal permeability. Amer. Jour. Bot., 2:93-94.

_____, 1915 e. On the nature of antagonism. Science, n. s., 41:255-256.

_____, 1916. A dynamical theory of antagonism. Amer. Phil. Soc., 55:533-555.

_____, 1917. Antagonism and permeability. Science, n. s., 45:97-103.

_____, 1919. Antagonism between alkaloids and salts in relation to permeability. Jour. Gen. Physiol., 1: 515-519.

Overton, E. F., 1895. Ueber die osmotischen Eigenschaften der lebenden Pflanzen - und Tierzelle. Vrtljh. Naturf. Gesell., Zurich. 40:159-201.

_____, 1899. Ueber die allgemeinen osmotischen Eigenschaften der Zelle, ihre vermutlichen Ursachen und ihre Bedeutung für die Physiologie. Vrtljh. Naturf. Gesell., 44:88-135. (Cited by Höber).

_____, 1900. Studien über die Aufnahme der Anilinfarben durch die lebende Zelle. Pringsheim Jahrb. Wiss., Bot., 34:669-701.

_____, 1901. Studien über Narkose, zugleich Beitrag zur allgemeinen Pharmakologie, 195 p., Fischer, Jena.

Palgen, W. B., 1925. Essai sur la biologie de quelques bacteries. These, Fac. Med., Nancy. (Cited by Buchanan and Fulmer, Vol. II, p. 337).

Peterson, E. E., 1927. A study of the preparation of syrups. Doctoral thesis, Library, Iowa State College, Ames, Iowa.

Prayleski, S. J., 1931. Effect of addition of salts on the isoelectric point of proteins I. Biochem. Jour., 25:715-727.

Rahn, O., 1928. Incomplete sterilization of food products due to heavy syrups. Canning Age, Aug., 1928, 705-706.

_____, 1929. The size of bacteria as the cause of the logarithmic order of death. Jour. Gen. Physiol., 13:179-205.

_____, 1932. Physiology of bacteria. 438 p., P. Blakiston and Sons, Philadelphia.

_____, and Barnes, M. N., 1928. Experimental comparison of the different definitions of death. Jour. Bact., 23:46-47.

Reichel, H., 1909. Zur Theorie der Desinfektion I. Abhandlung. Die Desinfektionswirkung des Phenols. Biochem. Ztschr., 22:149-251.

Reiter, R., 1920. Über die Gewinnung resistenter Milzbrandsporen. Arch. Hyg., 89:191-201.

Rideal, E. K., 1930. A system of bacteriology. Vol. I, 119-141, His Majesty's Stationery Office, London.

Robertson, A. H., 1927. Thermophilic and thermoduric microorganisms, with special reference to species isolated from milk.
I. Review of literature. N. Y. (Geneva) Agr. Expt. Sta., Tech. Bul., 130.
II. The thermal resistance of microorganisms. Vermont Agr. Expt. Sta., Bul., 274.
III. Description of non-spore forming thermoduric organisms isolated. N. Y. (Geneva) Agr. Expt. Sta., Tech. Bul., 131.
IV. The effect of age of culture on heat resistance. Vermont Agr. Expt. Sta., Bul., 275.

Rochaix, A., 1918. Sur la théorie de la désinfection par les agents chemiques. Compt. Rend. Soc. Biol., 72:333-334.

Rosenblatt, M., and Rosenblatt, M., 1910. Influence de la concentration en saccharose sur l'action paralysante de certains acides dans la fermentation alcoolique. Compt. Rend. Acad. Sci., (Paris), 150-1363-1366.

Russell, H. L., and Hastings, E. G., 1900. Thermal death point of tubercle bacilli under commercial conditions. 17th Ann. Rept., Wis. Agr. Expt. Sta., (1899-1900), 147-170.

_____, and _____, 1902. On the increased resistance of bacteria in milk pasteurized in contact with air. Centbl. Bakt., II Abt., 8:462-469.

Schulze, E., 1891. Über das Verhalten der Lupeinenkeimlinge gegen distillirtes Wasser. Landw. Jahrb., 20:855-(Cited by Hibbard).

Schumacher, J., 1929. Das Ektoplasmas der Bakterien. Zur Gramschen Färbung und zur Chemie der Desinfektion. Centbl. Bakt., I Abt., Orig., 107:161-180.

Seliber, G., and Katznelson, R., 1927. Sur l'influence de la composition du milieu sur la valeur osmotique de la cellule de levure. Compt. Rend. Soc. Biol., 97:449-450.

Seligman, E., 1933. Die Bedeutung der Wasserstoffionen-konzentration für die Prüfung von Desinfektionsmitteln. Centbl. Bakt., I Abt., Ref., 74:481-489.

Shaughnessy, H. J., and Falk, I. S., 1924. The effects of some electrolytes on the buffering capacity of Bacterium coli. Jour. Bact., 9:589-599.

_____, and Winslow, C.-E. A., 1927. The diffusion products of bacterial cells as influenced by the presence of various electrolytes. Jour. Bact., 14: 69-99.

Shearer, C., 1916-1917. On the toxic action of dilute pure sodium chloride solutions on the meningococcus. Roy. Soc., (London), Proc., Series B., 89:440-445.

_____, 1920. Studies on the action of electrolytes on bacteria I. The action of monovalent and divalent salts on the conductivity of bacterial emulsions. Jour. Hyg., (London), 18:337-360.

Sherman, J. M., and Albus, W. R., 1924. The function of lag in bacterial cultures. Jour. Bact., 9:303-305.

Sieffert, G., 1914. Die Abtötung pathogener Keime unter Glycerineinwirkung. Centbl. Bakt., I Abt., Orig., 74:644-650.

Smith, T., 1899. The thermal death point of tubercle bacilli in milk and other fluids. Jour. Expt. Med., 4:217-233.

_____, 1908. Some neglected facts in the biology of the tetanus bacillus. Jour. Amer. Med. Assoc., 50: 922-924.

Sommer, H. W., 1930. Heat resistance of spores of Clostridium botulinum. Jour. Infect. Diseases, 46: 85-114.

Spaeth, R. A., 1916. The vital equilibrium. Science, n. s., 43:502-509.

Standard Methods of Milk Analysis, 1929. Amer. Pub. Health Assoc., New York.

Stiles, W., and Jorgensen, I., 1917. Studies in permeability IV. The action of various organic substances on the permeability of the plant cell and its bearing on Czapek's theory of plasma membrane. Ann. Bot., 31:47-76.

Sumner, F. B., 1911. Fundulus and fresh water. Science, n. s., 34:829-831.

Süpfle, K., 1914-1916. Über die Resistenz der Bakterien und ihre experimentelle Prüfung. Sitzungsber. Ges. Morphol. Physiol., München, 30:42. (Abstract in Centbl. Bakt., I Abt., Ref., 67:329-330, 1919). Original not seen.

_____, and Dengler, A., 1915. Die Bedeutung optimaler Nährböden zur Nachkultur bei der Prüfung von Desinfektionsverfahren. Arch. Hyg., 85:189-197.

_____, and Müller, A., 1920. Über die Rolle der Absorption bei der Einwirkung von Sublimat auf Bakterien. Arch. Hyg., 89:351-354.

Toulouse, J. H., 1929. The action of citric acid and its salts in sugar solutions. Doctoral thesis, Library, Iowa State College, Ames, Iowa.

Traube, J., and Somogyi, R., 1921. Zur Theorie der Desinfektion. Biochem. Ztschr., 120:90-99.

True, R. H., 1915. The harmful action of distilled water. Amer. Jour. Bot., 1:255-273.

Viljoen, J. A., 1926. Heat resistance studies II. The protective effect of sodium chloride on bacterial spores heated in pea liquor. Jour. Infect. Diseases, 39:286-290.

Wallace, C. I., and Tanner, F. W., 1931 a. The effect of concentrated salt and sugar solutions on the thermo-death times of molds. Jour. Bact., 21:32.

_____, and _____, 1931 b. The effect of heat on mold spores. Soc. Expt. Biol. and Med., Proc., 28:970-972.

Watkins, J. H., and Winslow, C.-E. A., 1932 a. The effect of cell concentration and age of culture upon the mortality of bacteria exposed to alkalinity and heat. Jour. Bact.,23:51.

_____, and _____, 1932 b. Factors determining the rate of mortality of bacteria exposed to alkalinity and heat. Jour. Bact., 24:245-266.

Weil, R., 1899. Zur Biologie der Milzbrandbacillen. Arch. Hyg., 35:356-402.

Weiss, H., 1921 a. The heat resistance of spores with special reference to the spores of B. botulinus. Jour. Infect. Diseases, 28:70-92.

_____, 1921 b. The thermal death point of the spores of B. botulinus in canned foods. Jour. Infect. Diseases, 29:363-368.

Winslow, C.-E. A., and Brooke, C. R., 1927. The viability of various species of bacteria in aqueous suspensions. Jour. Bact., 13:255-243.

_____, and Cohen, B., 1918. Relative viability of B. coli and B. aerogenes types in water. Jour. Infect. Diseases, 23:52-59.

_____, and Dolloff, A. F., 1923. Relative importance of additive and antagonistic effects of cations upon bacterial viability. Jour. Bact., 15:67-93.

_____, and Falk, I. S., 1918. Studies on salt action II. The effect of transfer from stronger to weaker solutions upon the viability of bacteria in water. Soc. Expt. Biol. and Med. Proc., 15:131-134.

_____, and _____, 1923. Studies on salt action VIII. The influence of calcium and sodium salts at various hydrogen ion concentrations upon the viability of Bacterium coli. Jour. Bact., 8:215-236.

_____, and Haywood, E. T., 1931. The specific potency of certain cations with reference to their effect on bacterial viability. Jour. Bact., 22:49-69.

Wolff, A., 1908. Zur Kenntnis der Veränderungen in der Bakterienflora der frischen Milch während des sogenannten Inkubationsstadiums. Centbl. Bakt., II Abt., 20:651-675, also 757-780.

Wright, J. H., 1917. The importance of uniform culture media in bacteriological examination of disinfectants. Jour. Bact., 2:515-546.

Wright, H. N., and Hirschfelder, A. D., 1930. Studies on the colloidal chemistry of antisepsis and chemotherapy IV. The duplication in vitro of the "interference phenomenon" in combination chemotherapy. Jour. Pharmacol., 39:39-57.

v. Wunsheim, O., 1901. Beeinflusst Glycerin als Lösungsmittel den Desinfectionswerth von Antisepticis? Arch. Hyg., 39:101-141.

Zeug, M., 1930. Aequilibrierte Salzlösungen als indifferente Suspensionsflüssigkeiten für Bakterien. Arch. Hyg., 89:178-190.

Ziegler, K., and Dörle, M., 1930. Bakterienschutz gegen Metallsalze durch Gewöhnung. Ztschr. Ges. Expt. Med., 72:178-196.

Zoond, A., 1927. The interpretation of the changes in electrical resistance accompanying the death of cells. Jour. Bact., 13:9-10.

ACKNOWLEDGMENTS

For timely counsel, advice, and encouragement, an expression of sincere appreciation is hereby extended to Dr. B. W. Hammer, Dr. Ellis I. Fulmer, and Dean R. E. Buchanan of the Iowa Agricultural Experiment Station, and to Dr. L. D. Bushnell and Dr. F. L. Gainey of the Kansas Agricultural Experiment Station.