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EMERGENCE OF HEAT RESISTANCE IN POPULATIONS OF SALMONELLA SENFTENBERG

by

John Anthony Watson

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Environmental Health Sciences)
in The University of Michigan
1973

Doctoral Committee:

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Professor Lloyd L. Kempe

DEDICATION

To my wife, Dolores; my children--Susan, Martha, Robert, Caroline and Helen; my father, Arthur; my mother, Rose; and my aunt, Helen Landrigan.

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INTRODUCTION

Salmonellosis in man is primarily a foodborne disease and one of the most important communicable disease problems in the United States as well as in other countries of the world (van Oye, 1964). The National Research Council reports that an estimated 2,000,000 human cases occur annually in the United States with a total cost to the American economy of at least $3,000,000 in prevention, control, and therapeutic measures and in the financial losses suffered by associated industries and infected individuals. The disease is very difficult to control in man because the causative agent is widely distributed in nature and has access to him by a variety of routes (NRC Report, 1969).

Until the source problems of salmonellosis are resolved there is need for a constant vigil on the final protective barrier that is provided by adequate heating of potentially hazardous products normally consumed in the cooked state. Applied research is an important part of this vigil for there is a need to assist regulatory agencies in defining minimum "time-temperature" treatments required to assure destruction of salmonellae as well as other infectious pathogens and toxic microbial metabolites commonly associated with foods and their products.

When strains of Salmonella are found to exist which are so resistant to heat that they are purposely ignored in government regulations dealing with minimum "time-temperature" requirements for cooking, then research of a basic nature is needed to determine how these strains emerge from cell populations of a bacterial genera that is normally sensitive to heat, what gene product(s) is responsible for increased heat resistance, and in what manner a "normal" gene can be altered to produce such a product. This is more than an academic quest since such knowledge could be helpful in preventing other organisms from becoming heat-resistant, especially if it can be shown that this unwanted property can be induced by a single environmental factor or some combination of factors acting in concert. Such is the case with two reported heat-resistant strains of Salmonella senftenberg, S. senftenberg 775W (Winter et al., 1946), and S. senftenberg D8 (Davidson et al., 1966). Both these strains have been isolated from food and are capable of surviving a minimum heat treatment of 140°F for 3.5 minutes in liquid egg products (Winter et al., 1946; Annelis et al., 1954; Osborne et al., 1954; and Sugihara et al., 1966) and a minimum terminal temperature of 160°F in turkey rolls (Milone and Watson, 1970).

The fundamental problem to be resolved in studying the emergence of heat-resistant strains of Salmonella senftenberg is whether this property is nongenetic or genetic in origin. Nongenetic or natural changes are quantitative variations in

the phenotype of an organism that are within the potential limits of expression of the corresponding gene. Genetic changes are quantitative or qualitative variations in the phenotype of an organism due to the presence of an altered or newly acquired gene. Genes can be altered by spontaneous or induced mutations and can be acquired through the genetic transfer mechanisms of transformation, conjugation, and phage-mediated transduction or conversion.

This study was undertaken to determine if the unique property of heat resistance possessed by the Winter (SS775W) and Davidson (SSD8) strains of Salmonella senftenberg can be attributed to genes acquired by one of the mechanisms of gene transfer that is mediated by bacteriophage. The specific objectives of this study are:

1) To determine if the known heat-resistant strains of Salmonella senftenberg are lysogenic and carry related prophages,
2) To isolate a nonlysogenic, heat-sensitive strain of Salmonella senftenberg that can serve as a recipient host strain for the phages of SS775W and SSD8,
3) To recover bacteriophages from one of the heat-resistant strains in sufficient numbers to conduct genetic transfer experiments,
4) To determine if a genetic determinant for thermal resistance can be transferred by phage particles from a heat-resistant donor strain to the heat-sensitive recipient strain, and

5) To determine if the phage-mediated transfer mechanism for this genetic determinant is conversion or transduction.

The accomplishment of these objectives should determine the role of bacteriophage in the emergence of heat-resistant strains in populations of Salmonella senftenberg.

HISTORICAL

Variations in the Genus Salmonella

Types of Variation. Bacteria are variable rather than constant with respect to their morphological and physiological properties. These variations may be of genetic origin indicating that the organism is expressing a newly acquired or altered gene (genotypic variation) or they may be of natural (nongenetic) origin in which case the phenotype of the organism is quantitatively changed within the potential limits of expression of the corresponding gene (phenotypic variation). Examples of natural variation in organisms include the changes that occur in enzyme activity, are associated with the various phases of growth, and that take place between members of the same clone (Wilson and Miles, 1964; Davis et al., 1968).

Such a simple scheme for classifying changes that occur in microorganisms, however, is complexed with other dichotomies. Variations, for example, are also classified as being adaptive or nonadaptive, depending on whether or not they result from natural or induced changes in complex ecosystems and express the ability of the species to survive in a changed environment. In addition, they may also arise through the process of mutation and selection or be due to the presence of extra-genetic material originating in a host donor cell and transferred to a susceptible recipient cell by one of the three

recognized mechanisms of gene transfer-transformation (transfer of free or "naked" DNA--Griffith, 1928; Dawson, 1931; Alloway, 1932; and Avery, MacCleod, and McCarty, 1944); conjugation (transfer of genetic material following "cell-to-cell" contact or mating--Lederberg and Tatum, 1946a, b); and transduction (transfer of genetic material mediated by bacterial viruses--Lederberg, Lederberg, and Zinder, 1951; Zinder and Lederberg, 1952).

In the case of gene transfer mediated by a bacterial virus (bacteriophage or phage), a distinction is made between whether an altered phenotype is due to the bacterial genes carried by the infecting phage particle or is brought about by the genes of the normal virion. If the alteration can be determined to be under genetic control of the phage the phenomenon is called phage conversion rather than transduction (see Barksdale, 1959; Hartmann and Goodgal, 1959; Hayes, 1964, and Marmur et al., 1969).

Most cells capable of transferring their genetic material or portions of their genetic material have been shown to contain specific elements called episomes (see Hayes, 1964; Wilson and Miles, 1964; Davis et al., 1968; and Smith et al., 1968). Included among the elements classified as episomes are the F or fertility factor of Escherichia coli (Arber, 1960), the RTF factor of shigellae which is responsible for the transfer of multiple drug resistance (Watanabe, 1963), the temperate prophage of Salmonella typhimurium phage P22 (Zinder and

Lederberg, 1952) and the colicinogenic factor "C" (Fredericq, 1965).

Jacob and Wollman (1958b, 1961) define the episome as "a genetic structure which is added to the genome of a cell and which inside this cell may exist (and propagate) in two distinct and mutually exclusive states: the autonomous cytoplasmic (or extra-genomic) state and the integrated (intragenomic) state." Episomes that integrate into the bacterial genome do so either at one particular site (specialized integration) or at different sites (generalized integration) and changes or shifts of these elements from the integrated to the autonomous state, or vice versa, can alter the cells phenotype. An important property of episomes is that in the integrated state they can participate in rare genetic exchanges with neighboring regions of the host's genome and incorporate host genes into their own structure which can subsequently be transferred to other cells causing hereditary changes.

Natural Variation. When several colonies from one strain are tested, Salmonella species can be shown to display both biochemical and antigenic variations.

Biochemical variants have been reported for various species giving atypical reactions with lactose, maltose, inositol, glycerol, d-tartrate, glucose (gas production), H_2S and several ammoniated organic compounds (Kauffmann, 1966). No doubt other biochemical variants exist.

Salmonellae possess somatic and flagellar antigens and may possess envelope and fimbrial antigens which can vary in content

both qualitatively and quantitatively. The various types of antigenic variation known to occur in members of this group may be summarized as follows (see Kauffmann, 1966; Edwards and Ewing, 1962).

A. Form Variation

H-O variation. Transition of forms which contain both O and H antigens to forms containing only O antigens. Loss of the H antigens is irreversible and occurs rarely.

S-T-R variation. Transition of smooth forms to rough forms (S-R) or smooth forms to transient "T" forms that are smooth in appearance (S-T) with loss in both cases of O antigens characteristic of the strain.

O, Vi, and V-W variation. These are quantitative changes in O or V_i antigen. Various species have been found to lose quantities of the O antigens 1, 19, 24, 25, 27, 36, and 37 and the 6_1 partial fraction of O antigen 6 and 12_2 fraction of O antigen 12. V-W variation is exemplified by Salmonella typhi which has been found by agglutination tests to exist in three forms depending on its content of V_i antigen and its ability to agglutinate in specific O antiserum. Form "V" contains V_i antigen and is not O agglutinable; Form "W" does not contain V_i antigen and is O agglutinable; Form "VW" contains V_i antigen but is also O agglutinable.

M-N variation. Change of a mucoid form to the normal nonmucoid form with subsequent loss of the "M" envelope antigen.

F-NF variation. Change of a fimbriated form to a nonfimbriated form.

B. Phase (H) Variation

Phase variation is a definitive, qualitative change in flagellar antigens of which there are two types, monophasic and diphasic, depending on whether these antigens occur in one or two phases. If the phenomenon known as S-R phase variation is included we can list four kinds of phase variation:

Specific-non-specific phase variation (Andrewes, 1922, 1925). Variations occurring between a particular phase 1 antigen and antigens found in combination with another antigen (other than the e, n complex) in phase 2; for example, b:1,2; b:1,6; etc. or b:1,2 and i:1,2.

Andrewes (1922) found that serum prepared from colonies of two related types of Salmonella- S. typhimurium (1,4,5,12 : b;1,2) and S. paratyphi B (1,4,5,12 : i;1,2) - would agglutinate either both Salmonella types (heterologous serum) or only one type (homologous serum) in high dilution. Colonies that agglutinated only in homologous serum he called the "specific" phase of the organism and

those that agglutinated in both homologous and heterologous sera the "nonspecific" phase. It was later found that the antigen(s) responsible for the "specific" phase is not confined solely to any one particular type of Salmonella, as Andrewes had implied, but often occurs in other types in combinations with different O antigens or different "nonspecific" antigens. The Salmonella Subcommittee (1940) cognizant of this fact changed the nomenclature of Andrewes from "specific" phase to "phase 1" and the "nonspecific" phase to "phase 2."

α-β phase variation (Kauffmann and Mitsui (1930). Variations occurring between particular phase 1 antigens, such as b, and the antigens found in combinations with the e,n complex in phase 2; for example, b:e,n,x; b:e,n,z_{15}; etc. This type of phase variation is similar to that of Andrewes.

The phase variation of Edwards and Brunner (1938). Variations occurring between the 1,w complex in phase 2 and antigens in phase 1; for example, b:1,w; e,h:1,w; z:1,w; etc. Normally the 1,w complex is found only in phase 1. Organisms exhibiting this special type of "α-β" phase variation may be regarded as having two alternate phases.

S-R phase variation (Kauffmann, 1929a). Variations occurring between normal S phase antigens found in fresh isolates and those that arise spontaneously and are selected from media containing immune sera. In the R phase the normal S antigens may be completely or partially lost and replaced by new antigens. By growing S. wichita (1,13,23:d;-) in phase d antiserum, for example, Edwards et al. (1954) were able to isolate the flagellar antigen z_{37} which they considered to be a phase 2 antigen (d;z_{37}). When the organism (the R phase variant) was grown in z_{37} antiserum, however, it converted to the S phase and the change was irreversible. The R phases, first reported by Kauffmann (1929) for S. typhi, are referred to in the literature as the "induced phase" or "hybrid phase." Only the normal S phases for salmonellae are listed in the Kauffmann-White Schema. Kauffmann's S phase is also identical to the "irreversible" phase of Edwards and Ewing (1962). The antigenic formula for S. wichita may be written as 1,13,23:d↔z_{37};-to indicate it has an R phase.

Genetic Variation. In the genus Salmonella, a variety of nutritional, regulatory, fermentative, antigenic, and resistance markers have been transferred from donor strains to recipient "wild type" or auxotrophic mutant strains of similar species and to other species and related genera by the mechanisms of conjugation and phage-mediated transduction and

conversion. Claims for possible DNA transformation in this genus have been reported (see Hartmann and Goodgal, 1959) but the evidence offered has been ambiguous (Iino and Lederberg, 1964).

With the two recombination systems offered by the mechanisms of transduction and conjugation it has been possible to study the microtopology and macrotopology of the Salmonella chromosome by applying the transduction system for analysis of individual and closely linked genes and the conjugation system for mapping genes associated along the chromosome (Demerec and Hartman, 1959; Falkow et al., 1962; Miyake, 1962; Iino and Lederberg, 1964, Sanderson and Demerec, 1965; and Sanderson, 1967). In addition, both systems for recombination have been employed in studying many of the phenomena described as "form" and "phase" variation in the salmonellae with the result that our knowledge of the underlying biochemical and genetic mechanisms of these phenomena have increased (see reviews by Beale and Wilkinson, 1961; Iino and Lederberg, 1964; and Lüderitz et al., 1966).

The first definitive proof that a bacterial virus could transfer genetic material from one strain of bacteria to another was provided by Zinder and Lederberg (1952) who used the term "transduction" to describe the mechanism. These workers isolated a temperate phage called PLT-22 (also referred to as P-22) from Salmonella typhimurium LT-22, a strain they had obtained along with other lysogenic Typhimurium (LT) strains from the collection of Lilleengen (1948).

Using phage P-22 and U.V. induced auxotrophic mutants of S. typhimurium LT-22, S. typhimurium LT-7, and S. typhimurium LT-2 (an indicator strain for P-22), they were able to transduce various nutritional markers (methionine, histidine, tryptophan, proline, leucine, and phenylalanine and tyrosine), fermentative markers (galactose, xylose, mannitol and maltose), and resistance to the drug streptomycin. They were also able to transduce the "i" flagellar antigen from S. typhimurium to S. typhi with the formation of a new serotype. Phage P-22 is identical or very similar to the heat-resistant A1 phage of Boyd (1950). It attaches to strains having somatic antigen 12 (Salmonella "O" groups A, B, and D) and inserts into the S. typhimurium chromosome between the proline A and C cistrons (Smith and Levine, 1965).

Phage conversion of certain somatic antigens in the various subgroups of Group E Salmonella have been demonstrated and extensively studied by the Japanese workers. All Salmonella assigned to Group E share antigen 3 as a major antigen and are divided into subgroups on the basis of their content of other somatic antigens. Subgroup E_1 is characterized by somatic antigens, 3, 10; Subgroup E_2 by antigens 3, 15; Subgroup E_3 by antigens 3, 15, 34; and Subgroup E4 by antigens 1, 3, 19.

Iseki and Sakai (1953) are credited with first reporting antigenic conversion in bacteria. They demonstrated that E_1 strains of Salmonella could be converted to E_2 by infecting

them with a temperate phage they called ε^{15}. Subsequently, other investigators (Uetake et al., 1955, and Harada, 1959a,b) isolated and characterized a phage they called ε^{34} which was obtained from an E_3 strain of Salmonella and found capable of converting E_2 strains to E_3. Uchida et al. (1960) explained the biochemical nature of the change from E_1 to E_2 as the replacement of the terminal sugar acetyl-galactose by galactose and the change from E_2 to E_3 as the addition of glucose to the terminal galactose sugar.

Reports of antigenic conversion in Salmonella Group E were quickly followed by experimental infections of representative members of Salmonella "O" groups or subgroups A,B,C_1,D, G,K, R, and T with temperate "converting" phages that carried genetic determinants for somatic factors 1, 6, 12_2, 14, 15, 27, 34, and 42_2 (see Lüderitz et al., 1966).

Transfer of genetic material by the mechanism of conjugation has been demonstrated between E. coli and Salmonella (Baron et al., 1959a; Miyake and Demerec, 1959; and Zinder, 1960) and between Salmonella species (Baron et al., 1959b). Some of the markers that have been successfully transferred by this mechanism include those for H antigens (Mäkelä, 1964), O antigens (Mäkelä, 1965, 1966; Mäkelä and Mäkelä, 1966) and the S,R,S-R, and T antigens (Naide et al., 1965 and Sarvas and Mäkelä, 1965). Transfer of these markers have brought about changes in either the "phase" or "form" of the organism.

By means of conjugation, the salmonellae have also been shown to transfer such episomal elements as the "F factor-

chromosome fragment" factor F' (Baron et al., 1959b, and Ørskov et al., 1961), the multiple resistance transfer factor RTF (Watanabe, 1963; Sanderson and Lewis, 1965; Smith, 1966; and Gill and Hook, 1966), and various colicinogenic factors (Ozeki et al., 1962, and Smith and Stocker, 1962).

Definition and Characterization of the Species Salmonella senftenberg

Taxonomic Classification. Kauffmann working at the Robert Koch Institute in Berlin reported (1929b, 1930b-d) isolation of a new serotype member of a group of organisms he referred to as the "Paratyphoid Group." The organism had been recovered by him from the stool of an 8-year-old male patient who had been admitted to a Berlin hospital with symptoms of food poisoning. Kauffmann demonstrated the organism to have an antigenic formula of 1, 3:g,s; - and called it "Typhus-Senftenberg." He noted that while this organism shared a somatic serofactor 1 antigen with "Typhus A" (i.e., Salmonella paratyphi A), it did not resemble this group member in its biochemical reactions; notably, the new serotype, in contrast, fermented dulcitol, rhamnose, and xylose, and produced H_2S.

At about the same time Warren isolated what he and his colleague Scott thought was a new Salmonella serotype from the feces of a healthy carrier suspected of infecting and causing the death of her three children (Warren and Scott, 1930). The organism recovered from the mother, however, differed both antigenically and biochemically from the one that

was recovered from the third fatal case and was also different from other Salmonella species with which it was compared. Warren and Scott named their isolate Salmonella newcastle. Among the distinguishing biochemical characteristics of this organism was its ability to produce only a very feeble amount of H_2S. Kauffmann (1931), in examining the organism, found it to be antigenically identical to his "Typhus-Senftenberg" and named it "Typhus-Senftenberg-Newcastle."

The Salmonella Subcommittee (1934) recognized Kauffmann's isolate as a new but illegally named species having the antigenic formula 1,3:g,s: - and condensed the legal name of Salmonella senftenbergensis proposed by Haupt (1932) to Salmonella senftenberg. Warren's isolate was recognized by the Subcommittee as being antigenically identical but biochemically distinct from that of Kauffmann's and they assigned it the name Salmonella senftenberg var. newcastle.

In addition to its somatic 1 and 3 antigens and its phase 1 flagellar antigens, Salmonella senftenberg has been shown to possess a somatic 19 antigen and another phase 1 antigen, a "t" antigen. Phase 2 flagellar antigens for this organism have not been demonstrated (Kauffmann, 1941). The complete antigenic formula for Salmonella senftenberg, therefore, is 1,3,19: g,s,t; - . Because of the serofactor 19 antigen, which it shares with other serotypes of Salmonella, it has been assigned to Salmonella "O" Group E_4 in the Kauffmann-White Schema (Edwards and Ewing, 1962).

Distribution. The distribution of Salmonella senftenberg is like that of any other "unadapted" strain of Salmonella. It has been found in human carriers (Bornstein and Saphra, 1942; Edwards and Brunner, 1942; Olitsky et al., 1956) and apparently healthy pigs (Varela et al., 1942, 1952; Newell et al., 1959) and chickens (Smith and Buxton, 1951a,b). It has been isolated from human cases (Kauffmann, 1929b,c and 1930b-d; Warren and Scott, 1930; Savage, 1932; Hormaecke et al., 1939, 1943; Seligmann et al., 1943; Edwards and Brunner, 1942, 1943; Taylor, 1962, 1965; Yuruck and Best, 1965) and also from symptotic animals such as chickens (Edwards, 1939; Edwards et al., 1948; Watanabe et al., 1952; Pomeroy and Grady, 1960; Taylor, 1962, 1965; Moran et al., 1965; Boring, 1965), turkeys (Edwards, 1937; Edwards et al., 1948; Taylor, 1962, 1965), pigs (Moran et al., 1965; Taylor, 1962, 1965); cattle and other bovine species (Moran, 1965; Taylor, 1962, 1965), and ducks (Clarenburg and Vink, 1950).

Man has become infected with serotype Senftenberg through a variety of foods and food products. Included among these are Canadian egg products (Thatcher, 1965) and processed foods (Yuruck and Best, 1965); Chinese dried egg (Bornstein and Saphra, 1942); American, spray-dried egg (Seligmann et al., 1943; MRC Report, 1947; Solowey et al., 1947) and retail pork and beef (Cherry et al., 1943); frozen, raw beef exported from South America and Australia (Hobbs, 1965); and from dried, inactive Brewer's yeast used as an ingredient in tube-feeding

formulas prepared for hospital patients (Kunz and Ouchterlony, 1955).

It might be said that man, on occasion, paradoxically infects himself with this organism through the complete foods he gives his domesticated animals and from the ingredients used in their preparation. S. senftenberg has been isolated from South American beef and horsemeat used as animal food (Taylor, 1962, 1965; Hobbs, 1965), poultry feed (Pomeroy and Grady, 1960), pig feed (Newell et al., 1959), and various other contaminated raw materials (Taylor, 1962, 1965; MH Bulletin, 1961) such as meat meal (Galbraith et al., 1961), and crushed bone (Harvey and Price, 1962).

Prevalence. In various reports on the prevalence of Salmonella species and serotypes recovered from human and other sources, S. senftenberg has consistently placed among the top 35 cultures most frequently isolated. It ranked 20th in Kelterborn's report of a worldwide survey of Salmonella cultures recovered from human and other sources in 39 countries between 1936 and 1964 (Kelterborn, 1967) and, in reports on isolations from human sources in the United States and its territories, it ranked 30th in the 10-year period from 1948 to 1958 and between 25th to 32nd for the period from 1963 to 1967 (Edwards, 1965; Martin and Ewing, 1969).

Biochemical Characterization. Kauffmann (1966) lists the following biochemical reactions for Salmonella senftenberg:

Positive reactions. Arabinose, dulcitol, rhamnose,

trehalose, xylose, d-tartrate, citrate, and mucate.

Negative reactions. Inositol and gelatin.

Variable reactions. H_2S, glycerol, l-tartrate, and i-tartrate.

It should be noted that all reactions reported by Kauffmann were carried out at 37°C and that "positive" reactions for sugars and organic acids usually occurred in 24 hours. Negative reactions were recorded for sugars incubated for 30 days, organic acids for 14 days, gelatin for 90 days, and glycerol (Stern's glycerol fuchsin medium) for 8 days.

Earlier, Kauffmann (1961) had listed the biochemical reactions for S. senftenberg and S. senftenberg var. newcastle separately. The biochemical variant differed from S. senftenberg by failing to ferment glycerol, produce H_2S, and utilize i-tartrate. Reactions of S. senftenberg in i-tartrate, however, were usually delayed two to three days. In contrast, the reactions of the variant in i-tartrate medium were "positive" in 24 hours while those of S. senftenberg were "late and irregularly positive or negative."

Based on its ability to ferment dulcitol and mucate and its inability to liquefy gelatin, Salmonella senftenberg is classified as a member of Biochemical Subgroup I of the genus Salmonella (Kauffmann, 1966). Typical biochemical reactions of this subgenera which are not included along with the Kauffmann-White Schema are the following:

Positive Reactions. Sorbitol, maltose, mannitol (acid and gas), glucose (acid and gas), NH_4 glucose, NH_4citrate, KNO_3 reduction and methyl red.

Negative Reactions. Adonitol, salicin, lactose, sucrose, indole, acetylmethylcarbinol, urea and KCN.

Kauffmann, however, has found some strains of S. senftenberg that ferment sucrose and some that fail to produce gas from glucose.

Antigenic Structure. Salmonella senftenberg (S. enteritidis serotype 1,3,19:g,s,t; -) has been assigned to Salmonella O-Serogroup E in the Kauffmann-White Schema because it contains O-serofactor 3 as its major antigen and to subgroup E_4 because it has O-serofactors 1 and 19 as additional antigens. The O-antigens of Group E Salmonella all contain the sugars rhamnose and mannose in addition to the sugars which comprise the common basal core (i.e., - KDO, L-glycero-D-mannose, glucosamine, galactose and glucose) and have been chemically classified by Kauffman as Salmonella Chemotype XIII along with members of Group F which contain only serofactor 11 and with Group 54 which contain only serofactor 54.

Hapten inhibition studies by Staub and Girard (1965) and Uchida et al. (1963) have shown that a-D-glucose, a-D-galactose, a-D-mannose, and L-rhamnose are the immuno-dominant sugars of Group E_4 polysaccharides. The Group E_4 determinant is composed

of repeating trisaccharide units containing O-a-D-galactopyranosyl-(→6)O-a-D-mannopyranosyl-(1→4)-L-rhamnopyranoside with a secondary side chain of O-a-D-glucopyranosyl- in 1,6 linkage with the galactose residue. The O-specific side chain is attached to the common basal core through the rhamnose portion of the oligosaccharide.

The specificity of the O-E_4 antigens have been found to be associated with various combinations of the immunodominant sugars. Staubbs and Girard, for example, have established a relationship between O-serofactor 19 and the tetrasaccharide a-D-glucose-(1→6)-galactose-(1→6)-mannose-(1→4) and between the disaccharide a-glucose-(1→6)-galactose and serofactor 1. These investigators, together with Uchida and his co-workers, have also indicated a relationship between the trisaccharide mannose→rhamnose→galactose and the specificity of serofactor 3.

Group E_4 Salmonella species are identified by their qualitative content of flagellar antigens which in some serotypes appear in either one of two phases. Included in the "phase 1" antigens of this group are the antigens a, b, d, r, y, z, z_{29}, and some of the antigens comprising the eh complex (eh), the G complex (f, g, t; g, s, t; and mt), the L complex (l, v; 1, z_{13}; 1, z_{13}, z_{28}), and the z_4 complex (z_4, z_{23}). The "phase 2" antigens of the E_4 group are z_6, z_{35} and certain antigens of the en complex (en, z_{15}), the L complex (l, w), and the 1 complex (1, 2 and 1, 5). Kauffmann combines the z_6 antigen with the 1 complex in his pools of H antisera.

Most of the organisms in group E_4 are diphasic with the exception of S. westerstede (1, 3, 19:1, z_{13}; -), S. llandorf (1, 3, 19:z_{29}; -), S. cannstatt (1, 3, 19:m, t; -), S. dessau [(1), 3, 15, (19) :g, s, t; -], and S. senftenberg (1, 3, 19:g, s, t; -). In fact, the majority of the presently recognized types of Salmonella containing g or m antigens in phase 1 are monophasic (Kauffmann, 1966). To identify the various serotypes of Salmonella it has been necessary to isolate the organism in an actively motile form, test for the presence of antigens in one of the phases (usually phase 2), and then immobilize this phase with the appropriate immune antiserum in order to recover and analyze for antigens in the opposite phase. Furthermore, it has been standard practice in various Salmonella Reference Centers to test, by the same immune suppression or immobilization technique, cultures that are apparently monophasic.

Salmonella senftenberg exhibits the special type of phase variation that Kauffmann (1929, 1966) calls the "R-S" type and which Edwards and Ewing (1962) refer to as the "irreversible" type. Edwards et al. (1947) in reexamining a culture isolated from "normal" feces and identified by Edwards and Brunner (1942) as Salmonella simsbury (1, 3, 19:z_{27}; -) found that this organism changed to S. senftenberg (1, 3, 19:g, s, t; -) when it was grown repeatedly in semi-solid medium containing z_{27} immune serum. Evidence to support the contention that the z_{27} antigen was actually a phase 2 antigen of S. senftenberg was

indicated by the fact that the change from z_{27} to g, s, t was, for all practical purposes, irreversible. Only once did the reverse change from g, s, t to z_{27} occur and this was forcibly accomplished, with great difficulty, by repeated transfer in medium containing g, s, t antiserum. S. simsbury has been recovered with low frequency from animals as well as man and in reviewing the literature it is important to know the relationship between this organism and S. senftenberg. As a note it might be mentioned that the biochemical reactions of S. simsbury are identical to those for S. senftenberg rather than S. senftenberg var. newcastle, with the exception that i-tartrate is not utilized (see Kauffmann, 1951, 1954, 1961).

Edwards and Ewing (1962) have isolated and/or identified other strains of S. senftenberg containing z antigens which spontaneously change to g, s, t but do not revert back to their z forms (Taylor et al., 1960). These phase 2 serotypes of S. senftenberg (1, 3, 19:z_{43}; -, 1, 3, 19: z_{45}; -, and 1, 3, 19: z_{46}; -, are employed in the production of z_{27}, z_{43}, z_{45}, and z_{46} specific antisera. Kauffmann considers the phase containing the transformable antigens, such as the z antigens, the R-phase of the organism and the phase containing the irreversible antigens as the S-phase.

Heat Resistant Strains of Salmonella

With the enactment in June, 1965 of the new USDA regulation on egg pasteurization (see Federal Register, January 1, 1967) came a renewed interest in the heat-sensitivity of salmonellae

(Ng, 1966). Despite the fact that heat-resistant strains of Salmonella were known or reported to be in existence at that time, pasteurization under the new regulation was designed to eliminate only salmonellae of "normal" heat-resistance. One of these heat-resistant strains was Salmonella senftenberg 775W (NTCC No. 9959). This strain was isolated sometime between 1943 and 1944 from powdered egg and was found by Winter and his co-workers (1946) to be 10 to 20 times more heat-resistant than other strains of S. senftenberg and other Salmonella species tested in pasteurization experiments with liquid whole egg. Another strain suspected at that time to have heat-resistant properties was the TM-1 strain of Salmonella typhimurium. Husseman and Buyske (1954) reported this strain to have unusually high resistance to heat when it was suspended and thermally processed in ground chicken muscle.

Ng (1966), in an attempt to detect other heat-resistant strains of Salmonella, screened 300 cultures (representing 75 different serotypes) for heat sensitivity and compared the thermal-resistance of these strains with S. typhimurium TM-1. His experiments indicated that S. senftenberg 775W was 26 times more resistant, S. blockley 2004 was five times more resistant, and the majority of the remaining strains were 1.2 times more resistant than the TM-1 strain and six other strains of S. blockley. These results, as well as those of Bayne (1966), failed to confirm the alleged heat-resistance of the TM-1 strain.

Davidson and his colleagues (1966) have reported another heat-resistant strain of S. senftenberg which appears to be the first United Kingdom isolate. It was obtained in England from home-killed meat and found to be comparable in heat resistance to the 775W strain. Both were reported to be ten times more heat-resistant than other strains of Salmonella tested. Among these were 34 cultures of S. senftenberg isolated in various parts of the world; 20 of them were from human sources.

Cellular Basis for Heat Resistance

The cellular basis for heat resistance in nonsporulating bacteria is not definitely known but can be derived from studies of the biochemical differences between thermophilic organisms and their mesophilic counterparts in nature.

Thermophilic microorganisms are organisms that have the capacity to grow at temperatures above 50°C. Earlier studies on thermophiles have been reviewed by Gaughran (1947), Allen (1953), and Koffler (1957) and indicate that the most common explanation for thermobiosis has been that the essential components of thermophiles are more heat stable than those of mesophiles. More recent reviews on the physiological basis for thermophilism have been written by Farrell and Rose (1967) and Campbell and Pace (1968) and indicate which of the essential components of a vegetative bacterial cell determines its inherent thermal resistance.

The inherent property of thermal resistance possessed by spores and some bacterial cells is determined from survivor curves (log% survivors vs time) which are constructed from "thermal reduction time" or "thermal death time" data. A comparison of these curves for various organisms under different environmental conditions indicates that the thermal resistance of bacterial cells is influenced by a number of factors which include the age of the cell and the physicochemical nature of the medium in which they are grown, heated, and recovered. The nature of the heating menstruum is particularly important and environmental factors such as pH, water content, and the presence of certain organic and inorganic substances in various concentrations have been found to increase or lower the thermal resistance of an organism. As one might expect, studies of this nature invariably led to conclusions or speculations regarding the mode of action of heat on the bacterial cell. For a comprehensive review of the subjects of survivor curves and their interpretations and factors which have been found to influence the thermal resistance of microorganisms, see the reviews by Schmidt (1957), Hansen and Riemann (1963), Stumbo (1965), Prost and Rieman (1967) and Brown and Melling (1971).

Allwood and Russell (1970) have recently reviewed and commented on the various mechanisms postulated for thermal injury and death of nonsporulating bacteria by moist heat. These mechanisms include damage to the cell wall or membrane, damage to the nuclear material, coagulation of proteins or inactivation

of enzymes, and degradation of ribosomes and ribosomal RNA (rRNA). In their commentary, these authors make note of the mounting evidence suggesting a direct correlation between degradation of ribosomal RNA in vegetative bacteria and loss of cell viability during storage at sublethal temperatures and the limited direct evidence offered to support any of the other mechanisms. The concept that degradation of rRNA is mainly responsible for injury and death of the cell is reinforced by the finding that regeneration of rRNA is essential for its recovery (Iandolo and Ordal, 1966 and Sogin and Ordal, 1967).

Campbell and Pace (1968), in their review, discuss the work that has been done to determine the heat stability of ribosomes and rRNA of various organisms in relation to thermal denaturation and protein synthesis. With some exceptions, studies in which thermal denaturation profiles and melting temperatures (T_M) were determined for ribosomes and rRNA of selected psychrophilic, mesophilic, and thermophilic bacteria (Pace and Campbell, 1967 and Saunders and Campbell, 1966) showed (a) a direct positive correlation between the melting temperature of the ribosomal particles examined and the maximum growth temperatures for these organisms, and (b) that the melting temperature for rRNA is lower than either the maximum growth temperatures of the organism or the melting temperature of its ribosome. Saunders and Campbell (1966) found no significant difference between the T_M profiles of the 16S and 23S rRNA fractions from E. coli and B. stearothermophilus which

indicated that the greater heat stability of the thermophile was not due to an unusual rRNA species. An investigation by Pace and Campbell (1967) to determine a relationship between the maximum growth temperature of an organism and its total content of guanine and cytosine (G+C content) in rRNA and DNA revealed that there is a slight increase in the G+C content in rRNA with increasing growth temperatures but that no such correlation exists with DNA. In addition, their study indicated no apparent relationship between the G+C content of rRNA and DNA. This apparent lack of correlation between the gross base composition of rRNA and heat denaturation of the intact ribosomes, together with the other findings mentioned above, led Campbell and Pace to speculate that the thermal stability of the ribosomes rested either in the fine structure of the rRNA-protein interaction or in differences in the primary structure of the ribosomal proteins.

Other evidence that ribosomes of thermophiles are more heat stable than those of mesophiles comes from studies on protein synthesis. Friedman et al. (1967, for example, were able to demonstrate that ribosomes of B. stearothermophilus heated at 65°C for various periods of time retained a much higher activity in supporting polynucleotide-directed amino acid incorporation under ideal conditions than ribosomes of E. coli heated at the same temperature. Experiments with similar results were carried out by Algranati and Lengyel (1966).

MATERIALS AND METHODS

Test Organisms

Almost all of the organisms employed in this study were strains of *Salmonella senftenberg*. These strains were obtained from various sources and were assigned an identifying "SS" number as they were received in the laboratory. Strains that were not identified by this number were the 775W strain and D8 strain of *Salmonella senftenberg* and the LT-2 strain of *Salmonella typhimurium*.

Stock cultures of all strains were maintained as 18-hour - 35°C, slant cultures on L-Agar No. 2 medium held at 4°C; 18-hour - 35°C, stab cultures in TSA No. 2 medium held at 4°C; and as cell suspensions in PBG solution held at -20°C. The PBG cell suspensions were prepared from overnight slant cultures. Each month, stock slant cultures of the organisms under test were prepared in duplicate. One culture from each pair was used to make daily working cultures and the other was kept sealed and used for the next monthly transfer. Stock cultures other than the stock slants served primarily as "back-up" cultures for the entire collection of organisms.

The following is a brief description of the organisms used in this study which includes mention of the source of the culture and the strain identifying number(s), source of the

organism with reference to associated human and animal infection and disease, and particular characteristics of certain strains which are of special interest to this study and require emphasis:

S. senftenberg Strain 775W. This strain was obtained from Dr. Herbert E. Hall, Chief of Food Microbiology, National Center for Urban and Industrial Health, Public Health Service, Department of Health, Education, and Welfare, Cincinnati, Ohio. NCUIH obtained the strain from Dr. M. Solowey, Army Research Laboratories, Fort Detrick, Maryland.

The 775W strain is the first reported heat-resistant strain of Salmonella. It was isolated sometime between 1943 and 1944 from powdered egg, has no associated history of human illness, and was found by Winter (1946) to be more heat-resistant than other Salmonella species in pasteurization experiments with liquid whole egg. This strain has been deposited in the National Collection of Type Cultures, Central Public Health Laboratory, Colindale, England and is identified in this collection as NCTC strain No. 9959.

S. senftenberg Strain D8. This strain was obtained from Dr. C. M. Davidson, Bacteriology Section, Unilever Research Laboratory, Sharnbrook, Bedford, England.

The D8 strain was originally isolated in England in 1965 from home-killed meat. Davidson et al. (1966) reported it to be comparable in heat resistance to the 775W strain and apparently the first heat-resistant Salmonella isolated in the United Kingdom. It was not associated with human illness (Davidson, 1967).

S. senftenberg Strain SS3. The SS3 strain was obtained from Dr. K. Stanley Read, Virology and Developmental Microbiology Section, Michigan Department of Health, Lansing, Mich. It was identified as MDH isolate No. SW2136 but no description of the source of the organism was given.

S. senftenberg Strains SS4 to SS63. These strains were obtained from Dr. W. H. Ewing, Chief of Enteric Bacteriology Unit, Bacteriology Section, National Communicable Disease Laboratory, Public Health Service, United States Department of Health, Education, and Welfare, Atlanta, Georgia. For NCDC number and description of source see Table I.

S. senftenberg Strain SS64. This strain was obtained from Dr. J. E. Williams, Research Veterinarian, United States Department of Agriculture, Agricultural Research Service, Southeast Poultry Research Laboratory, Athens, Georgia. The source of the strain was given as avian but it was not identified with an ARS number.

S. senftenberg Strains SS65 to SS87. These strains were obtained from Dr. Joan Taylor, Salmonella Reference Laboratory, Central Public Health Laboratory Service, Colindale, London, England. For the Colindale number and description of source see Table II.

S. typhimurium Strain LT-2. This strain was obtained from Dr. Myron H. Levine, Department of Human Genetics, The University of Michigan, Ann Arbor, Michigan. This organism is the indicator and propagating strain for the generalized transducing phage P-22.

TABLE I

IDENTIFICATION OF SALMONELLA SENFTENBERG STRAINS OBTAINED FROM THE NATIONAL COMMUNICABLE DISEASE CENTER IN ATLANTA, GEORGIA

Strain No.	Source	NCDC No.
SS4	Ala. - human female, feces	5111-63
SS5	La. - human female, 39 yrs	5043-63
SS6	Ala. - human female, 54 yrs, feces - case	4338-63
SS7	N.C. - human female, 3 yrs ,feces	4523-64
SS8	Ariz.- human female, 62 yrs, feces	5886-64
SS9	N.D. - sewage	2325-64
SS10	Md. - human male - treated with antibiotics	3779-64
SS11	Mex. - human adult, feces	4614-64
SS12	Ore. - human female, feces	590-65
SS13	Wash.- dog	1265-65
SS14	Fla. - human female, 3 1/2 yrs - hospitalized with fever, treated with chloromycetin	5112-65
SS15	Mass.- water - Merrimac River Project	6369-65
SS16	N.J. - river water	991-65
SS17	N.C. - human female, feces	3209-66
SS18	N.J. - water	112-66
SS19	Ark. - human female, infant, feces	3420-66
SS20	Mich.- water	3307-67
SS21	Ohio - feed	514-67
SS22	CDC - turtles	713-67

TABLE I-continued

Strain No.	Source	NCDC No.
SS23	Ore. - human male, adult, feces - diarrheal disease	1131-67
SS24	Tex. - meat and bone	5081-58
SS25	Ga. - poultry by-product	1621-61
SS26	N.C. - feed by-product	1629-61
SS27	Minn.- meat and bone	5199-61
SS28	Ga. - rendering plant	6753-61
SS29	Iowa - swine feces	771-62
SS30	Ky. - human male, feces	1160-62
SS31	Tex. - feed	3443-62
SS32	Fla. - human male	4291-62
SS33	Fla. - human	5053-63
SS34	Tenn.- human-asymptomatic	4289-63
SS35	Md. - human feces - diarrhea	3981-63
SS36	Va. - human, 1 mo, feces	2445-63
SS37	Vt. - human pus from gall bladder	4038-63
SS38	Fla. - human	2012-64
SS39	Utah - United by-product - discharge #3	3938-64
SS40	N.Y. - human, 23 mos, feces - 2 weeks of loose stools	3296-64
SS41	La. - human, 44 yrs, feces	5848-64
SS42	Pa. - human	1932-64
SS43	Wash.- dog	746-64

TABLE I - continued

Strain No.	Source	NCDC No.
SS44	N.Y. - human, 23 mos, feces	4058-64
SS45	Ala. - human, 22-25 yrs, feces - asymptomatic	1173-64
SS46	Fla. - human, feces	3935-64
SS47	Okla.- human,3 yrs, feces	1044-64
SS48	N.Y. - human, 23 mos, feces	4123-64
SS49	Ala. - human, feces	31-64
SS50	Fla. - human	134-64
SS51	Fla. - human	4305-64
SS52	Mich.- human, 21 yrs, feces	690-64
SS53	Okla.- human, 50 yrs, feces - diarrhea	5071-65
SS54	Fla. - human	2855-65
SS55	Ala. - human, 56 yrs, - diarrhea	4402-65
SS56	N.Y. - human, 3 yrs, feces	4190-65
SS57	Va. - human, feces	232-66
SS58	Iowa - human	2179-66
SS59	Ala. - human, feces - diarrhea and vomiting, no fever	1961-66
SS60	D.C. - liquid egg	917-66
SS61	Utah - meat	6102-66
SS62	Utah - bakery	4748-66
SS63	Ohio - water	4190-67

TABLE II

IDENTIFICATION OF SALMONELLA SENFTENBERG STRAINS OBTAINED FROM THE CENTRAL PUBLIC HEALTH LABORATORY IN COLINDALE, LONDON

Strain No.	Source	Colindale No.
SS65	Routine Virus Laboratory;Monkey	232-59
SS66	P.H.L. Brighton; Monkey	260-59
SS67	Medical Research Institute, Accra; African patient	1842-59
SS68	P.H.L. Kingston-upon-Hull, Yorks; Bone meal	1872-59
SS69	Food Hygiene Lab., Colindale; Coconut powder	1939-59
SS70	P.H.L. Chelmsford; Animal food	3505-59
SS71	Hillingdon Hospital, Uxbridge; G.D. Chainey (patient)	2120-59
SS72	Dept. Path., Shenley Hospital; John Greenhalgh (patient)	422-61
SS73	Min. Ag. Fisheries and Food; Intestines of six-week-old turkey	194-67
SS74	Metropolitan Water Board, Redbridge Works; Sewage effluent	465-67
SS75	P.H.L. Chelmsford, Essex; "Fidomeat" dog food	477-67
SS76	Dept. Vet. Bact. and Hygiene, Punjab Ag. University, India; Wall lizard	512-67
SS77	Shipping Federation, South Shields Alton D'souza (patient)	948-67
SS78	P.H.L. St. Luke's Hospital, Guilford, Surrey; Bone meal	960-67

TABLE II - continued

Strain No.	Source	Colindale No.
SS79	St.Andrew's Hospital, London; Xidhas Andreas (patient), diarrhea following operation for hiatus hernia	1024-67
SS80	P.H.L. Hull; Polluted sea-water from Durban, South Africa	1670-67
SS81	P.H.L. Maidstone; Sewer swab	3423-67
SS82	P.H.L. County Hall, London; Julian Sanz (patient with diarrhea)	3112-67
SS83	Unilever REsearch Lab., Bedford; No other information	2909-67
SS84	Unilever Research Lab., Bedford; No other information	2910-67
SS85	P.H.L. Neasden; C.R. Steel (male, 24 yrs)	2785-67
SS86	P.H.L. Maidstone; J.D. Coombs (patient)	2578-67
SS87	British Oil and Cake Mills; Indian meat meal	2556-67

Media and Reagents

The following materials were employed in this study:

BGA: Brilliant Green Agar (Difco* No. 0285).

Salmonella "O" Subgroup E_4 Antiserum: Salmonella "O" subgroup E_4 antiserum (Difco No. 2259-47).

C_1L-Broth: L-Broth with 1.0% sodium citrate.

C_2L, C_4L, C_8L-Broth: L-Broth with 2.0%, 4.0%, and 8.0% sodium citrate, respectively.

L-Agar (L-Agar No. 1): L-Broth with 1.5% Agar.

L-Agar No. 2: L-Broth with 2.0% Agar and omission of dextrose. Used in the preparation of agar slants.

L-Broth (L-Broth No. 1): 1.0% Tryptone; 0.5% Yeast Extract; 0.5% NaCl; 0.1% Dextrose. See Lennox (1955), and Smith and Levine (1965).

L-Broth No. 2: L-Broth with 0.1% $MgSO_4$ and 0.015% $CaCl_2$.

L-Broth No. 3: L-Broth with 1.0% $MgSO_4$ and 0.03% $CaCl_2$.

L-Broth No. 4: L-Broth with 1.5% $MgSO_4$ and 0.04% $CaCl_2$.

LSA No. 1 (L-Soft Agar No. 1): L-Broth with 0.75% Agar. Tubed in 3.5 ml quantities and used in assaying for bacteriophage by the method of soft agar "overlay" (SAO).

LSA No. 2 (L-Soft Agar No. 2): L-Broth with 0.7% Agar and 1.0% $MgSO_4$.

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*Difco Laboratories, Detroit, Michigan

TSA No. 2: Tryptic Soy Broth without dextrose (Difco No. 0862) to which 0.65% Agar is added. Used for the maintenance of stock cultures as stabs in "soft" agar at 4°C.

Phosphate Buffered Glycerol (PBG) Solution: A sterile solution containing 0.25% K_2HPO_4 and 0.5% NaCl in 60% glycerol. Used for the maintenance of stock cultures as cell suspensions in PBG at -20°C.

Assays for Bacteriophage

The soft agar "overlay" (SAO) method may be used quantitatively for the determination of bacteriophage by preparing the overlay with a mixed suspension of diluted phage and indicator bacteria or used qualitatively by spotting material containing the phage on overlays (lawn cultures) that contain only the indicator bacteria. The SAO method was used in this study to determine the titer of Salmonella bacteriophages in lysates and to detect Salmonella strains that were lysogenic. The method is a modification of the one described by Adams (1959).

Quantitative Test. Serial, decimal dilutions of bacterial lysates containing bacteriophage were prepared in L-Broth and added, in 0.4 ml quantities, to tubes containing 3.6 ml of melted and tempered LSA No. 1 or No. 2 medium. To each of these tubes was added 0.1 ml of a culture of an indicator strain of bacteria that had been grown in L-Broth at 35°C to log phase (1×10^7 viable cells per ml). The dilute, phage-bacteria suspensions were thoroughly mixed and poured into Petri dishes

containing 20 ml of hardened L-Agar. The plates were held at room temperature for 20 minutes to permit adsorption of the phage and then inverted and incubated at 35°C for 16 hours. Following incubation, plates containing between 30 to 300 plaques per 0.4 ml of dilute phage suspension were used to determine the titer of the lysate. The titer was recorded as plaque-forming units per ml (PFU/ml).

Qualitative Test. For the qualitative test the overlay was prepared in the same manner as described above but contained only the indicator strain of bacteria. The test was carried out with either cell-free lysates or lysates containing non-viable cells which were spotted onto the surface of the overlay (lawn culture) with a capillary dropping pipette or a bacteriological loop. After incubation, the spotted areas were read and qualitatively scored (0-4+) for varying degrees of complete lysis and for the numbers of plaques present.

Screening Test for Salmonella Lysogens

The screening test for Salmonella lysogens was carried out with unfiltered supernatants of S. senftenberg cultures which were prepared by growing the test cells in L-Broth for 16-18 hours at 35°C and decanting and saving the clear broth after centrifugation for 15 minutes at 12,000xg. These supernatants were spotted on lawn cultures of selected strains of S. senftenberg prepared by the SAO method. After spotting, the test plates were allowed to dry for 20 minutes before inversion and incubation at 35°C for 16 hours. Scoring for the definite

presence of lysogens in the spotted supernatants was made only for those lawn areas which exhibited either confluent or semi-confluent lysis or contained more than 50 plaques. The strains employed for detecting Salmonella lysogens were:

1. SS775W and SSD8. These strains were used to detect others in the collection carrying phages unrelated to the heat-resistant strains.
2. SS5, SS7, and SS32. By previous testing, these strains were considered most likely to serve as lysogen indicators.
3. The LT2 Strain of Salmonella typhimurium. This strain is the normal propagating strain for the generalized transducing phage, P-22.

Preparation of Growth Curves

Growth curves for S. senftenberg 775W were determined by the "viable cell count" method and by the "turbidimetric" method.

A "standard" growth curve for this organism (log viable cells/ml vs. time) was constructed for cells suspended in L-Broth and grown at 35°C for 24 hours with constant shaking (Fig. 1). The suspension was made by inoculating 199 ml of L-Broth preheated to 35°C in 500 ml, screw-capped, Erlenmeyer flasks with 1 ml of a "seed" culture containing approximately 2×10^4 CFU/ml. The "seed" culture was a mid-log phase, broth shaker culture that was diluted in broth to give the desired cell concentration.

During incubation of the culture, 4 ml samples were removed with a bulb pipette at selected time intervals and analyzed for

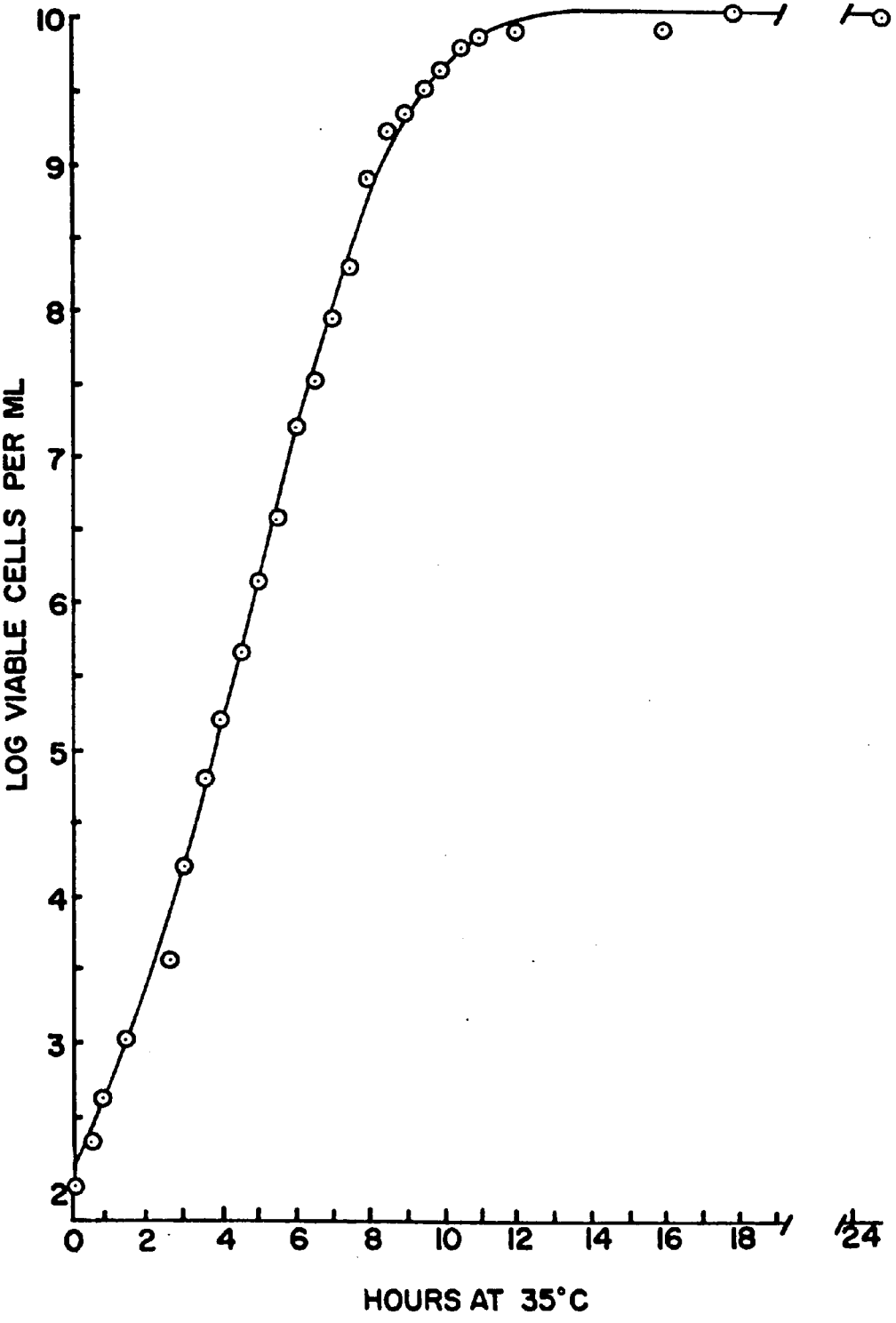


FiG. 1. Growth of Salmonella senftenberg 775W in L-Broth at 35 C.

turbidity and viable cell concentration (Figs. 2 & 3). Turbidity was measured with a B&L* Spectronic "20" at wavelenghts of 425 and 620 mμ using standard adsorption tubes (1/2 inch B&L colorimeter tubes) and were recorded both as percent transmittance (%T) and as optical density (OD) of the culture in relation to the growth medium. Viable cell concentrations were measured by plating broth dilutions of the sample in L-Agar and determining the number of colony-forming units appearing in the medium after 24 hours incubation at 35°C.

Turbidimetric growth curves (log %T vs. time and OD vs. time) were determined for the test organism during the late-log phase of growth where changes in turbidity could be measured with a spectrophotometer. Curves of this type were determined for growth of the organism in Erlenmeyer flasks (see above), nephelometer flasks (500 ml, nephelometer flasks with 14x100 mm sidearms; Bellco** No. 5201) and in calibrated test tubes ("matched," 16x125 mm, screw-capped, test tubes).

When nephelometer flasks were used, the inoculated medium was incubated with shaking until the turbidity of the culture, as measured in the sidearm tube, could be detected spectrophotometrically. At that time, and at selected intervals of time thereafter, turbidity of the culture was measured and recorded and 4 ml samples immediately removed to chilled test tubes for determination of viable cell concentration and for turbidity

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*Bausch and Lomb, Rochester, New York.

**Bellco Glass, Inc., Vineland, New Jersey.

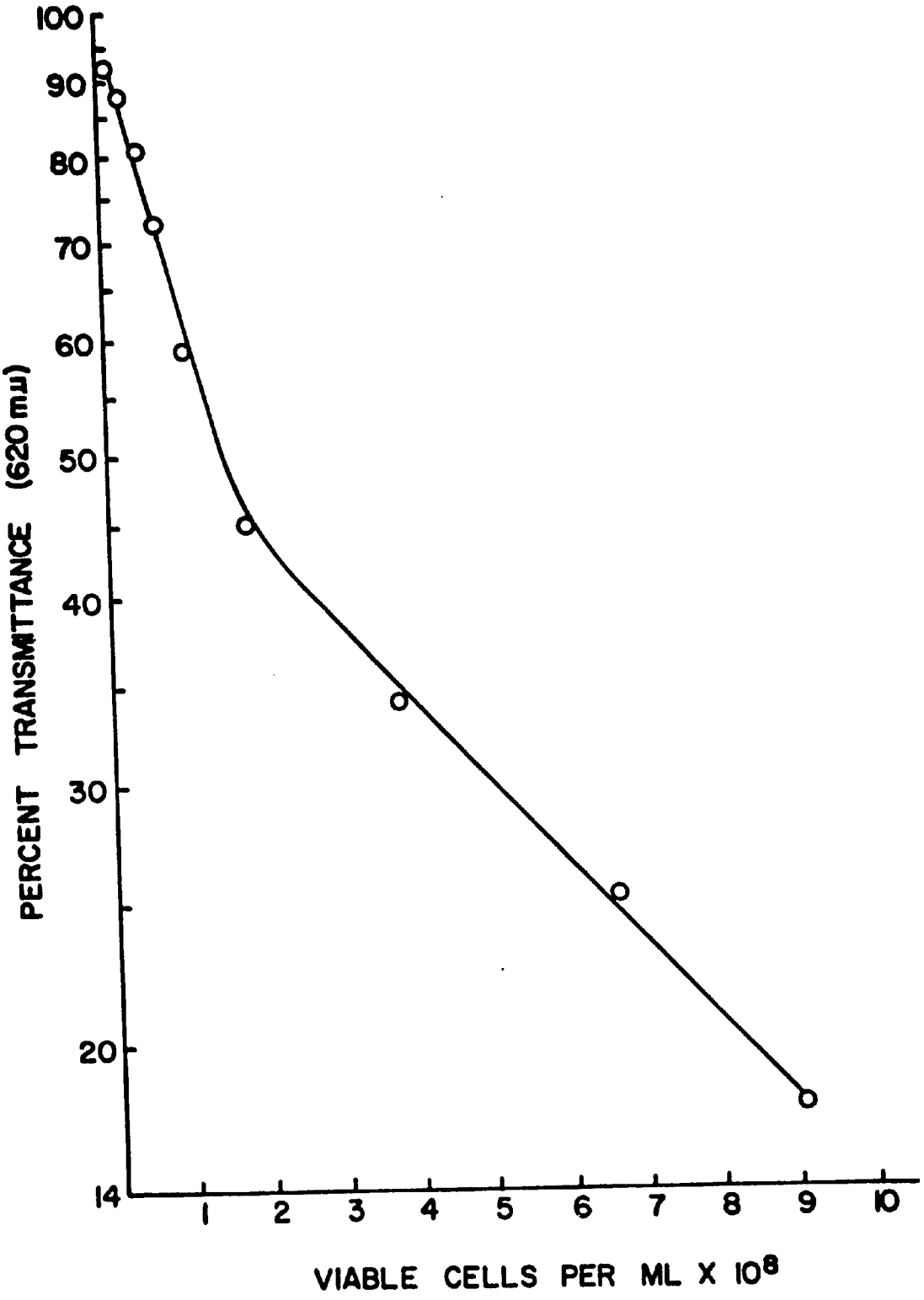


FiG. 2. Relationship between viable cell concentration and turbidity of Salmonella senftenberg 775W broth cultures at 620 mμ.

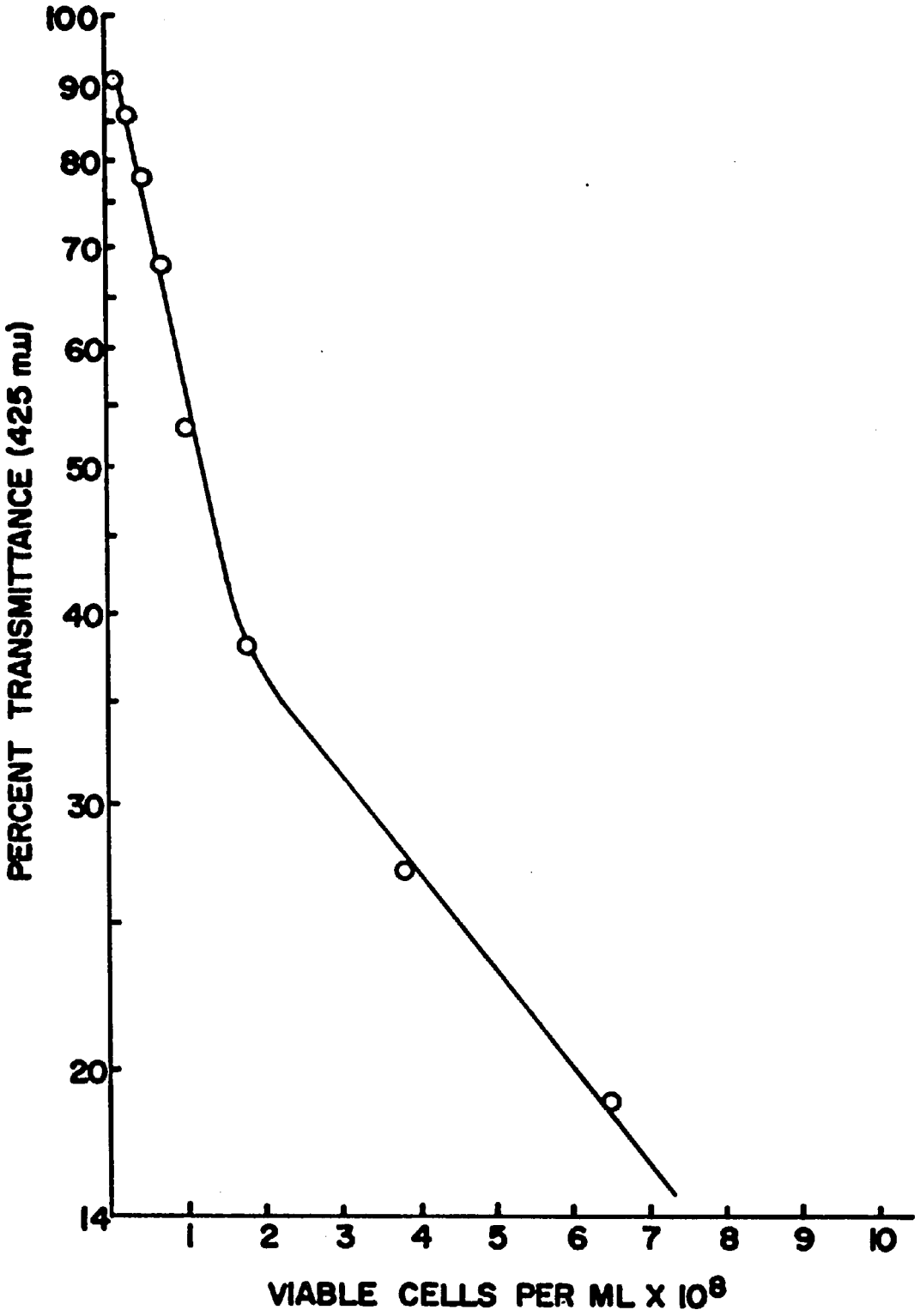


FiG. 3. Relationship between viable cell concentration and turbidity of Salmonella senftenberg 775W broth cultures at 425 mμ.

measurements in both calibrated test tubes and "standard" absorption tubes. With such information it was possible to determine the relationship between the turbidity of a culture growing in Erlenmeyer flasks, nephelometer flasks and calibrated test tubes and the viable cell concentration and the phase of growth of the organism.

Unless otherwise stated, all broth cultures in this study were prepared as shaker cultures at 35°C and dilutions of these cultures were made in L-Broth.

Preparation of Sterile Lysates

Sterile, ultraviolet induced (UVI) lysates were prepared from 50 to 100 ml of late-log phase broth cultures of SS775W contained in 500 ml nephelometer flasks. When the culture reached this phase of growth, 5 ml samples were quickly removed and placed into each of 10 to 20 plastic Petri plates (15x100 mm). These plates were irradiated, with swirling, under a 15-watt germicidal lamp fixed at a distance of 15.5 inches from lamp filament to the bottom of the dish. Use of a flat tray enabled the irradiation of 25 mls of culture at a single exposure to UV light. Following irradiation, the culture was returned to the flask, reincubated, and its subsequent growth, lysis, and regrowth monitored with the spectrophotometer, usually until the point of maximum lysis was reached. In comparative UVI studies, incubation was often continued beyond this point with 5 ml samples of the irradiated culture taken at informative midpoints and inflection points. For

these studies, spectrophotometric readings were made in a 35°C "walk-in" incubator.

Sterile lysates were prepared by centrifuging the lysed cultures at 8000 xg for 20 minutes at 4°C and serially filtering the supernatants through 0.45μ and 0.2μ cellulose triacetate membranes. Samples of supernatants and filtrates were removed and quantitatively assayed for phage by the soft-agar "overlay" (SAO) technique before storage of the lysate at 4°C.

Thermal Inactivation Studies

A "spinner bottle" method was used for conducting thermal inactivation studies. In general, this method consists of introducing 1 ml of a dilute, 24-hour, broth culture (approximately $1x10^{10}$ viable cells/ml) into a "spinner" bottle containing 99 ml of suspending medium preheated to a desired temperature. During heating of the suspension, 2- to 3-ml samples were quickly removed with a bulb pipette at predetermined time intervals and immediately transferred to chilled, sterile test tubes. These tubes were refrigerated until serial, decimal broth dilutions could be made of the samples for plating on recovery medium.

The "spinner" bottle is an 8-oz. graduated, prescription bottle containing a teflon-coated, spinning bar which is air-driven by a magnetic stirrer submerged and fixed in a well agitated water bath. The temperature of the bath was accurately controlled (±0.1°F) by a thermoregulator and relay monitoring system.

Screening Tests for Thermal Resistance

In general, cells from a 24-hour broth culture were diluted in broth to give an approximate final concentration (10^0 dilution) of either $1x10^8$ or $1x10^9$ viable cells per ml and then heated at 60°C for 2.5 or 3 minutes using one of the methods given below. The heated suspensions were diluted in broth and either inoculated as 1.0 ml "pour" plates or 0.1 ml "spread" plates in or on L-Agar to determine the number of survivors which were reported as colony-forming units (CFU) or spotted on L-Agar or BGA medium to determine colony-development patterns. Spotting was done with a bacteriological loop that delivered 1/230 ml of the cell suspension onto the surface of the agar medium.

Four methods of heating were used in this study to screen isolates for heat resistance--a "spinner bottle" method, a "sero-pipette" method, a "capillary pipette" method, and a "tube" method. Pipette methods were substituted for the "spinner bottle" method later in the study and consisted of heating cell suspensions in broth at 60°C for 2.5 minutes in pipettes that were heat-sealed at one end. Generally speaking, the "sero-pipette" method was used when the surviving heat-treated cells were to be enumerated in or on recovery medium and the "capillary pipette" method was used when they were only to be spotted and observed for colony development patterns.

"Spinner Bottle" Method. This method is the same as the one used in thermal inactivation studies with the exception that a single sample was removed after 2.5 minutes of heating at 60°C.

"Sero-pipette" Method. A dilute broth suspension of the test organism at a concentration of $1x10^9$ viable cells per ml was drawn to the 0.2 ml mark of a sterile, glass, disposable, cotton-plugged, one-ml serological pipette (Corning* No. 7078) and the volume (0.8 ml) brought to the "zero" mark by elevating the tip of the pipette to an angle of 30 degrees and regulating the backward flow of the suspension by releasing the holding pressure applied with the index finger. After filling, the tip of the pipette was wiped with gauze saturated with a 1 percent iodine-alcohol solution (1 gram of iodine crystals dissolved in 100 ml of 70% ethanol) with care being taken to avoid introducing the disinfectant into the pipette. The tip of the pipette was immediately sealed in the flame of an air-gas burner and then rapidly cooled between several layers of aluminum foil. When properly sealed, the cell suspension in the pipette never reached lethal temperatures as witnessed by the fact that, after heat sealing and foil cooling, the pipette could be comfortably held at the air-liquid interface near its tip.

After sealing, the content of the pipette was identified with a label of 1/4 inch masking tape, placed in a wooden support and then cooled in an ice-water bath. The pipette support was a 3/4-inch pine board having a single row of 10 holes that were slightly larger than the pipette and set 1/2 inch apart. The masking tape label was fixed near the open end of the pipette in the appearance of a flag and prevented the

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*Corning Glass Works, Corning, New York.

pipette from slipping through the hole. Use of this support allowed rapid insertion of ten sealed pipettes into and out of the water bath and, in addition, kept them separated during the period of heating.

In performing the screening test for heat resistance, the supported pipettes were removed from the ice-water bath and immersed in a 60°C water bath to a depth of two inches above the upper level of the cell suspension. After the heating period (2.5 minutes), they were then quickly removed from the water bath and cooled in the ice-water bath. Measured with a temperature recording potentiometer that was activated by a thermocouple in contact with the cell suspension, the "come-up" time from 5°C to 60°C was 20 seconds.

After heating and cooling, the pipettes were removed from the support and scored with a diamond-point pencil at a distance of approximately 3/8 inch from the sealed end.

To prevent contamination of the heated suspensions during their removal from the pipette, each pipette was immersed in 1 percent iodine-alcohol solution for 15 seconds, wiped with gauze soaked in the same type of disinfectant, immersed in ethanol to remove the iodine, and then aseptically wiped dry with sterile laboratory tissue.

In breaking the pipette, the sealed end was aseptically wrapped in sterile laboratory tissue and placed on a raised, flat-edged surface with the "scored" site at the edge and directed "upward." With the index finger of one hand tightly over the open end of the pipette and the thumb of the other hand

immobilizing the sealed end, the pipette was easily broken at the "scored" site with a quick, downward movement of the open end.

After each pipette was broken, its content was delivered to a sterile, screw-capped test tube. This tube (called the "10^0 dilution" tube) was marked for identification of the organism tested and stored at 4°C until serial, decimal broth dilutions could be prepared for spotting and/or plating.

"Capillary Pipette" Method. The procedure for carrying out the screening test for heat resistance using the "capillary pipette" method is the same as that described for the "sero-pipette" method with the following exceptions:

a. Pasteur disposable pipettes with a total length of 230 mm and a capillary stem length of 120 mm are used instead of 1 ml, serological pipettes.

b. The cell suspension to be heated contains $1x10^8$ or $1x10^9$ viable cells per ml and the suspension is sealed in the stem of the Pasteur pipette between a 20 mm and 90 mm mark measured from the pipette tip.

c. No special support is needed to hold and separate the capillary pipettes. The bulb ends of several pipettes are held tightly together with a rubber band and are readily separated by the natural geometry of the pipettes themselves.

d. The end of the capillary pipette need not be scored for breaking. The sealed end of the pipette, after being

disinfected, is aseptically wrapped in sterile laboratory tissue and elevated so that the body length of the pipette makes a 30° degree angle with the table top. In this position it is easily broken between the thumbs and the index fingers of both hands without any loss of its contents. Before the pipette is returned to a horizontal position, however, the pipetting finger must be held tightly over the bulb end.

e. The contents of capillary pipettes were discharged directly onto marked areas of a Petri dish bottom. Spotting was done only with undiluted, heated cell suspensions.

Tube Method. In some screening tests the cell suspensions were heated at 60°C for 3 minutes in 16x125 mm, screw-capped, test tubes. In performing these tests, the tubed cell suspensions were preheated to 45°C prior to the test and during the test were immersed in the 60°C water bath to a depth of 1 inch below the cap and continuously rotated by hand to effect mixing of the suspension during heating. After the 3-minute heating period, the tubes were removed to an ice-water bath and rapidly cooled using the same method of agitation. The "come-up" time from 45°C to 60°C was 45 seconds.

To prevent contamination with portions of the tube and cap that were not immersed in the heating bath, the upper third portion of each of the tubes was heated in a flame for ten seconds and the original cap was replaced with a new one just prior

to the test. Immediately after flaming, the tubes were placed in an ice-water bath to prevent excessive heating of the cell suspension in the lower portion of the tube.

Heat Resistance Transduction Experiments

Attempts to transduce (or convert) a genetic marker for thermal resistance in salmonellae were carried out using S. senftenberg Strain 32 (SS32) as the recipient cell and S. senftenberg 775W phage (PSS775W) as the transferring particle. The methods and procedures that were used in this study to prepare SS32 lysogens containing SS775W prophage and recover "hr" transductants (i.e., organisms carrying transferred bacterial markers for heat resistance) differed to varying degrees and these differences are noted below in the description given for each experiment.

Preparation of SS32 Lysogens Carrying SS775W Prophage

The method used in the final stages of this study to prepare SS32 lysogens carrying genetic elements transduced by phages of the heat-resistant 775W Strain of S. senftenberg is as follows: SS32 cells were grown in L-Broth No. 1 on a 35°C shaker to a late-log concentration of approximately 2×10^8 CFU per ml and, following centrifugation at 8000 xg for 20 minutes at 4°C, resuspended in L-Broth No. 4 to the same concentration. The phage suspensions were sterile UVI lysates of S. senftenberg 775W in C_1L-Broth having a titer of approximately 2×10^8 PFU per ml. Calculated volumes of the bacterial suspension, L-Broth No. 4 and the phage suspension were mixed together in

a 40 ml centrifuge tube in the sequence given to give a total of $15x10^8$ phage and $5x10^8$ bacteria (m.o.i. = 3). The amount of L-Broth No. 4 added to the bacterial suspension was equal in volume to the phage suspension.

The PBM suspension (mixed suspension of phage and bacteria) was held at 20°C, with occasional shaking, for 30 minutes to allow for contact and adsorption of the phage and then diluted with an equal volume of L-Broth and centrifuged to obtain packed cells. After removal of the supernatant fluid, the cells were resuspended in 5 ml of L-Broth No. 1 and subjected to various treatments which were designed to recover "hr" transductants.

As a negative control for each transduction experiment, a bacterial suspension (B suspension) was prepared and treated in an identical manner as the PBM suspension with the exception that, instead of being mixed with lysate containing PSS775W phage, it was mixed with an equivalent amount of C_1L-Broth.

HRT Experiment No. 1

Preparation of the PBM Suspension. The PBM suspension for this experiment was prepared by the addition of 4 ml of SS32 cells in late-log phase that had been centrifuged and resuspended to a 2x concentration in L-Broth No. 2 (total CFU = $16x10^8$) to 4 ml of phage suspension (total PFU = $8x10^8$). Adsorption was carried out at room temperature and the cells in the PBM suspension were not centrifuged and resuspended in fresh broth.

Recovery of Transductants. A method of "cyclic" growth and thermal inactivation was used to select transductants with

thermal resistance markers from the test (PBM) suspension and to select survivors from the control (B) suspension. In this experiment the progeny of the cells in the original PBM and B suspensions survived 2 1/2 cycles of growth and heat inactivation (3 phases of growth and 2 phases of heat inactivation).

To start the growth phase of the first cycle, 3 ml samples were removed from the PBM suspension and transferred to each of two 500 ml Erlenmeyer flasks containing 97 ml of L-Broth No. 2. In like manner, two flasks serving as controls were inoculated with the B suspension. After all four flasks had been inoculated, they were divided into two sets with each set containing a control and a test cell suspension. One set was quickly brought to experimental temperature and incubated on a shaker at 35°C for 26 hours and the other was incubated on a shaker at room temperature (25 ± 1°C) for 32 hours. The flasks were labeled 25B, 25PBM, 35B, and 35PBM to identify the bacterial cells they contained with respect to growth temperature and their exposure or nonexposure to phage.

In the first heating phase, "spinner" bottles containing 99 ml of L-Broth preheated to 60°C were inoculated with 1 ml quantities of 29 ± 3-hour stationary phase cultures containing the 25B, 25PBM, 35B, and 35PBM cells (see above) and heated at this temperature for a period of 90 minutes. Samples were removed at 0, 2.5, 5, 7.5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, and 90 minutes of heating at 60°C and plated for survivors.

For the second cycle the inoculated bottles were removed from the water bath after 90 minutes of heating and incubated with shaking at the original growth temperature of the suspended cells. When the four cultures again reached a stationary growth phase (44 hours), one ml portions were removed to spinner bottles for thermal inactivation as before. Survivors were determined for samples removed at 0, 2.5, 5, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, and 90 minutes of heating at 60°C. Following the second heating, the bottles were incubated for 24 hours but samples were not removed to undergo a third heating. Samples taken during the two heating phases and the third growth phase were stored at 4°C and later examined for the presence of "hr" transductants.

Survivors were determined as "spread" counts on L-Agar plates incubated for 16 hours at 35°C and five days at 25 ± 1°C. In selecting survivors it was the general rule to pick colonies from those plates having between 3 to 30 colonies. By using this approach and picking all or a representative number of colonies from these plates it was felt that the "hr" transductants would eventually appear as the predominating survivor at some point in one of the cycles and be selected as an isolate.

Survivors selected for isolation were picked onto L-Agar (9 isolates per plate) and assigned both an isolate number and a corresponding treatment and recovery identification (TRI) number. After overnight incubation at 35°C the plates were sealed to prevent dehydration and stored at 4°C for future testing.

HRT Experiment No. 2

The method of "cyclic" growth and thermal inactivation was also used in a second controlled experiment to select transductants with thermal resistance markers from a mixed suspension of phage and bacteria. In this experiment the progeny of the cells in the original suspension were carried through three cycles of growth and thermal inactivation.

This experiment differed from the first HRT experiment in the following manner: (a) the cells were grown at 35°C only, and thermal inactivation was carried out at 55°C instead of 60°C; (b) in the first and second cycles, the heated cells were in the late-log phase of growth ($1x10^9$ per ml) instead of in stationary phase; and (c) the cells employed in the second and third phase of thermal inactivation represented the 0.01 percent survivors of the first and second phase of inactivation, respectively.

Preparation of the PBM Suspension. The PBM suspension for this experiment was prepared by adding 15 ml of a lysate containing PSS775W at a concentration of $1x10^8$ PFU per ml to a nephelometer flask containing 15 ml of a resuspension of SS32 cells in L-Broth No. 4 at a concentration of $0.33x10^8$ CFU per ml (m.o.i. = 3). The cells employed were from a culture in the late-log phase of growth. The PBM suspension was held at 20°C, with occasional shaking, for 30 minutes to allow for phage adsorption. In this experiment, the suspended cells were not centrifuged and resuspended in L-Broth after the adsorption

period. Bacterial suspensions were prepared in the same manner except for substitution of C_1L-Broth for the phage lysate.

Recovery of Transductants. Following the period of adsorption, the PBM suspension was diluted with 100 ml of L-Broth No. 1, quickly warmed to incubation temperature in a water bath and then incubated at 35°C on a shaker. During incubation, growth of the culture was monitored with a spectrophotometer at a wavelength of 620 mμ with turbidity readings observed and recorded at periodic intervals and compared with standard "turbidimetric" and "viable count" growth curves to determine cell concentration and phase of growth. When the culture was in the late-log phase of growth ($1x10^9$ cells/ml), a 10 ml sample was removed for thermal inactivation studies. Growth during this period represented the first phase of growth of the cells.

In the first phase of thermal inactivation a 1 ml sample of the late-log phase culture was inoculated into 99 ml of broth contained in a "spinner" bottle and heated at 55°C. During heating, 3 ml samples were removed at selected periods of time (0, 1, 2, 3, 4, 5, 7.5, 10, 15, 20, 25, and 30 minutes) and transferred to tubes chilled in an ice-water bath.

For each of the samples taken during thermal inactivation--a 1.1 ml portion was removed for plating to determine the number of survivors, a 1.0 ml portion was transferred to a tube containing 5 ml of broth and immediately incubated at 35°C, and the remainder of the sample was stored at 4°C.

By incubating portions of the samples taken during the first phase of inactivation it was possible to start the second cycle on the following day with a culture that originally contained a known number of survivors based on 24-hour plate counts. An attempt was made to use ".01 sample" cultures (i.e., overnight cultures of samples that contained approximately 0.01 percent survivors) throughout the experiment.

The method and procedure used for the second phase of thermal inactivation was identical to the first inactivation phase with the exception that samples were removed at 0, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 7.5, 10, 15, 20, 25, 30, 40, 50, and 60 minutes of heating at 55°C. As in the previous cycle, portions of the sample were removed for plating and for growth at 35°C and the remainder of the sample was stored at 4°C.

The third phase of thermal inactivation was carried out with a 26-hour, ".01 sample" culture which represented the third growth phase. The culture was diluted 1:10 in broth and 1 ml of this dilution was inoculated into 99 ml of broth contained in a "spinner" bottle and heated at 55°C. Samples were removed at 0, 4, 15, and 60 minutes of heating and plated for survivors.

Survivors were determined as colony counts on and in L-Agar after 24 and 48 hours incubation at 35°C and were recovered for further testing, as in the first HRT experiment.

HRT Experiments Nos. 3 and 4

HRT Experiment No. 3. The PBM suspension employed in the third HRT experiment contained 15×10^8 PFU of PSS775W and 5×10^8

CFU of SS32 cells in a total volume of 15 ml. The method used in the preparation and subsequent treatment of this suspension has been described (see "Preparation of SS32 Lysogens Carrying SS775W Prophage"). Following adsorption and centrifugation, the cells in the test (PBM) suspension and the control (B) suspension were resuspended in 5 ml of L-Broth No. 1, transferred to 16 mm, spectrophotometer tubes, and incubated with shaking at 35°C. When the number of cells in each of the suspensions had increased to a concentration of $1x10^9$ per ml (representing an increase of 3.32 generations) they were heated at 60°C for 3.0 minutes (see "Screening Tests for Thermal Resistance--Tube Method").

Following heating, each of the cell suspensions was spread onto the surface of 12 BGA plates which were allowed to dry before being placed in an incubator. The plates were held for 5 days at 45°C and 5 days at room temperature and examined daily for survivors.

Cellular concentrations in this experiment were determined from a "log %T vs. viable cell count" curve that was prepared for the calibrated 16 mm test tubes.

HRT Experiment No. 4. This experiment was conducted in the same manner as HRT Experiment No. 3 with the folling exceptions:

1. The test (PBM) suspension and the control (B) suspension were prepared in triplicate.
2. The centrifuged and resuspended cells from each of the three test and control suspensions were pooled

separately and then transferred, in 5 ml aliquots, to 16 mm spectrophotometer tubes. As in the third HRT experiment, the cells were grown at 35°C to a concentration of approximately $1x10^9$ per ml (3.32 generations) and then heated at 60°C for 3.0 minutes by the tube method.

3. After heating, the cells from the test and control suspensions were again pooled separately and 2 ml samples were removed from the two pooled suspensions and transferred to each of two sets of L-Agar plates (10 plates/set) and two bottles containing 50 ml of L-Broth. One inoculated bottle and set of plates was incubated for 48 hours at 45°C and the other at 35°C.
4. Survivors were determined as colony-forming units on L-Agar and as total turbidity-forming units in L-Broth.

All tests and control isolates recovered in these two experiments were stored at 4°C for further testing.

Biochemical Characterization Tests

The various tests employed in this study for the biochemical characterization of Salmonella senftenberg strains have been described in detail by Wetzler et al. (1968) and were performed in the laboratory of Dr. Wetzler when he was at the School of Public Health, The University of Michigan.

EXPERIMENTAL RESULTS

Lysogen Production in Strains of Salmonella senftenberg

In preliminary tests to determine if the heat-resistant strain, Salmonella senftenberg 775W, carried a prophage, it was observed that S. senftenberg strains SS5, SS7, and SS32 were lysed by crude lysates prepared from mixed cultures of SS775W and strains SS3 through SS43 and that strains SS7 and SS32 could serve to detect bacteriophage produced by SS775W.

The method used in these tests for lysogen detection was a modification of the one described by Atkinson (1957) in which a crude extract or lysate containing a phage from one of two bacterial strains was prepared by growing the strains in mixed culture on a small area of plated agar, removing the growth after overnight incubation at 37°C and suspending it in broth, and centrifuging the suspension to obtain the supernatant. The supernatant or crude lysate was then tested for lytic activity on both of the strains by placing a small spot of the lysate on to a small inoculated area of plated agar and examining the area for lysis after incubation. A positive lytic test indicated which one of the strains carried the lysogen and which one served to propagate it and could be used as an indicator to detect its presence.

Atkinson's method was modified in this study by growing the strains as a mixed culture in broth instead of on agar and carrying out the lytic tests with individual cultures prepared as soft agar overlays instead of as "patches" of cultures on the surface of agar plates.

When supernatants from 87 pure cultures of S. senftenberg, including the heat-resistant strains of SS775W and SSD8, and one culture of S. typhimurium LT-2 (STLT2) were spotted on SAO lawn cultures of SS5, SS7, SS32, SS775W, and STLT2, a little more than half the strains (58%) were shown to contain lysogens for one or more of these indicator strains by the method of testing and scoring employed (see Table III). These strains are as follows: SS775W, SSD8, SS3, -4, -5, -6, -8, -9, -11, -12, -14, -15, -18, -19, -21, -22, -23, -24, -25, -26, -28, -29, -31, -33, -34, -38, -42, -43, -44, -45, -47, -48, -49, -50, -55, -56, -59, -60, -66, -67, -71, -75, -79, -80, -81, -82, -83, -84, -85, -87.

Strains being reported as not containing lysogens for the indicator strains are: SS7, -10, -13, -16, -17, -20, -30, -32, -35, -36, -37, -39, -40, -41, -46, -51, -52, -53, -54, -57, -58, -61, -62, -63, -64, -65, -68, -69, -70, -72, -73, -74, -76, -77, -78, -86.

The presence of lysogens in the unfiltered supernatants was determined by their ability to lyse or form isolated plaques in lawn cultures prepared with the indicator strains.

In negative lytic tests, neither lysis nor plaques were observed in the lawn cultures areas lying beneath or immediately surrounding the surface colony that developed from bacteria

TABLE III

LYSOGEN PRODUCTION IN STRAINS OF SALMONELLA SENFTENBERG

Indicator Strain	Strains Producing Lysogens
SS775W	SS18, -24, -25, -26, -27
SSD8	SS24, -25, -26, -27, -84
SS5	SS11, -14, -21, -23, -24, -25, -26, -27, -43, -44, -47, -48, -49, -55, -59, -79, -80, -82, -85
SS7	SS775W, SSD8, SS11, -15, -19, -25, -28, -31, -34, -43, -47, -60, -67, -87
SS32	SS775W, SSD8, SS3, -4, -5, -6, -8, -9, -11, -12, -15, -18, -22, -23, -24, -25, -26, -27, -29, -31, -33, -38, -42, -45, -50, -55, -56, -59, -60, -66, -67, -71, -75, -81, -83, -84, -85
STLT2	SS75, -83

present in the unfiltered supernatant. These colonies appeared circular and flattened, had a raised edge, and were approximately one cm in diameter.

In positive lytic tests either lysis was observed around the surface colony or plaques were seen in the lawn culture beneath the colony. Positive lytic tests were reported as confluent (4+) or semiconfluent (3+) lysis around the colony or as greater than 50 (2+), 20 to 49 (1+), or less than 20 (±) plaques in the lawn culture beneath the colony. Lysis of the surface colony by lysogens in the lawn cultures could be recognized by either failure of the surface colony to develop into a typical colony or, in many cases, by the "moth-eaten" appearance of its edge. The lysogens appearing in Table III all gave at least a 2+ reaction with the indicator strains tested.

Four of the five Senftenberg indicator strains were found susceptible to L.SS24, L.SS25, L.SS26, and L.SS27 (i.e., lysogens carried by strains SS24, SS25, SS26, and SS27, respectively). The SS7 indicator strain was not susceptible to L.SS24, L.SS26 or L.SS27. Other general lytic reactions observed were as follows:

a) L.SS14, -21, -44, -48, -49, -79, -80, and -82 lysed only SS5.

b) L.SS19, -28, -34, and -87 lysed only SS7.

c) L.SS3, -4, -5, -6, -8, -9, -12, -22, -29, -33, -38, -42, -45, -50, -56, -71, -81, and 84 lysed only SS32.

d) L.SS23, -55, -59, and 85 lysed only SS32 and SS5.

e) L.SS775W, L.SSD8, L.SS15, -31, -60, and -67 lysed only SS32 and SS7.

f) L.SS75 and L.SS83 lysed only SS32 and STLT2.

g) L.SS18 lysed only SS32 and SS775W.

h) L.SS84 lysed only SS32 and SSD8.

i) No lysogens were found that could lyse both SS5 and SS7.

In this phase of the study it was shown that the heat-resistant strains, SS775W and SSD8, carried temperate phages for SS32 and SS7 and that, from all appearances, SS32 was non-lysogenic. SS32 was found to produce no plaques on any of the indicator strains employed and was lysed by 42.5 percent of the 87 strains of S. senftenberg tested. As a result of these experiments SS775W and SS32 were selected as the test strains for determining whether or not the character of heat resistance is a genetically transferable trait in salmonellae.

Induction of S. senftenberg 775 Prophage with Ultraviolet Irradiation: Factors Contributing to the Production of Sterile, High-Titer Lysates Containing PSS775W

In this study, a considerable amount of time was spent in optimizing some of the many factors that influence the production of sterile, ultraviolet induced (UVI) lysates of S. senftenberg 775W containing PSS775W in sufficient concentration for use in transduction experiments. The results of this study are discussed below.

Effect of Age of Irradiated Cells, Irradiating and Growth Medium, and Irradiation Dosage on Phage Yield. A series of experiments were conducted to determine the effect of the following parameters on phage yield:

a) Age of Irradiated Cells. The cultures employed were in the late-log phase of growth with varied cell concentrations between 2.0×10^8 and 8.4×10^8 per ml.

b) Irradiating and Growth Medium. The media studied were L-Broth and L-Broth without yeast extract.

c) Irradiation Dosage. Cells were exposed to ultraviolet light for various periods of time (5, 15, 20, 30, 45, and 60 seconds) under standardized laboratory conditions.

The results of this study indicated that the highest plague titers were obtained when L-Broth cultures having cell concentrations of $6.0 \pm 0.4 \times 10^8$ were irradiated for a period of 20 seconds.

Effect of Citrate on Phage Yield. In a pilot experiment to obtain SS775W phage for thermal reduction time studies it was found that a greater degree of lysis with subsequent higher plaque titers could be obtained by growing irradiated cells in L-Broth containing 1.0 percent sodium citrate (C_1L-Broth). As a result of this finding, experiments were designed to determine the maximum phage yield obtainable when varying concentrations of sodium citrate (1%, 2%, 4%, and 8%) were incorporated into the growth and irradiating medium. In some of these experiments a comparison was made between use of citrated medium

and addition of sodium citrate crystals to the irradiated culture at the beginning of lysis. The results obtained indicated that, although similar U.V. induction curves and higher titers were obtained by either pre- or post-addition of sodium citrate, the highest plaque titers were obtained when 1.0 or 2.0 percent sodium citrate was incorporated into the growth medium.

Effect of Mg^{++} on Efficiency of Plating. A divalent cation is needed by many phages for absorption and penetration. The following experiment was carried out to determine the requirement of PSS775W for Mg^{++}: A dilute phage suspension previously titered one day before and adjusted to give approximately 100 plaques in LSA No. 1 medium was retitered in the same medium containing 0, 0.1, 0.2, 0.4, and 0.8 percent $MgSO_4$. It was found that an increase in the $MgSO_4$ concentration from 0 to 0.8 percent produced a twofold increase in the plaque titer thus demonstrating that SS775W phage is stimulated by this divalent cation.

As a result of this experiment, LSA No. 2 medium was used routinely in assaying for PSS775W. It was felt that this medium gave the maximum number of plaque-forming units obtainable from phage suspensions and also plaques of sufficient size for counting. LSA No. 2 medium is the same as LSA No. 1 with the exception that it contains 1.0 percent $MgSO_4$ and 0.70 percent rather than 0.75 percent agar.

Sterilization of Phage Preparations. In transduction experiments it is absolutely essential that preparations are

not contaminated with the donor strain to avoid reaching the false conclusion that a particular marker has been transduced to the recipient. To prevent this from happening, supernatants of bacterial lysates must be sterilized. This can be accomplished, among other ways, either by the addition and removal of chloroform in a final concentration of 4 percent, or by use of membrane filters. There are disadvantages to both methods; in some cases the phage is susceptible to chloroform treatment and in other cases significant numbers of phage particles are retained by the membrane.

Both methods of preparing sterile phage preparations were studied and, of the two, membrane filtration was chosen to sterilize supernatants from SS775W lysates. Supernatants that were chloroformed and stored overnight at 4°C exhibited a significant decrease (ca, 90%) in plaque titer, indicating that PSS775W (like phage M13 of E. coli) is chloroform sensitive. In contrast, supernatants serially filtered through 0.45 μ and 0.2 μ cellulose triacetate membranes generally showed only a 20 percent loss in plaque titer. These filtrates were considered sterile since no growth was observed in tubes containing 5 ml samples of the filtered lysates after 10-day incubation at either 35°C or ambient temperature.

Production of Sterile, High-Titer, UVI Lysates of SS775W. Sterile UVI lysates of SS775W with acceptable phage titers for performing transduction experiments (ca, 2×10^8 PFU/ml) were obtained when mid-log phase cultures of SS775W were diluted in

C_1L-Broth to a final concentration of approximately $1x10^4$ cells per ml and irradiated for 20 seconds under standardized laboratory conditions after the cells had reached a late-log concentration of ca, $6.0 \pm 0.4x10^8$ per ml. Following irradiation, the culture was reincubated with shaking until maximum lysis was obtained, at which time the crude lysate was centrifuged and the supernatant recovered. Lysis began approximately 60 minutes after irradiation and reached its maximum at 105 minutes. Supernatants of UVI lysates were sterilized with very little loss in titer by membrane filtration. Typical turbidimetric growth curves for irradiated cultures of S. senftenberg 775W in citrated and noncitrated L-Broth and for an irradiated culture in citrated medium are shown in Figure 4. A similar curve for an irradiated culture of SS775W in citrated medium is compared in Figure 5 with those obtained for an irradiated and unirradiated SS32 culture.

Genetic Transfer Experiments for Heat Resistance

As soon as sterile, high titer SS775W lysates containing PSS775W became available, an attempt was made to transfer a genetic marker for heat resistance from SS775W, a heat-resistant strain of Salmonella, to the SS32 strain which, from lytic and UV induction tests and preliminary screening tests for thermal resistance, appears to be nonlysogenic and heat-sensitive.

Four experiments were carried out to select organisms having a transferred marker for heat resistance. The procedure

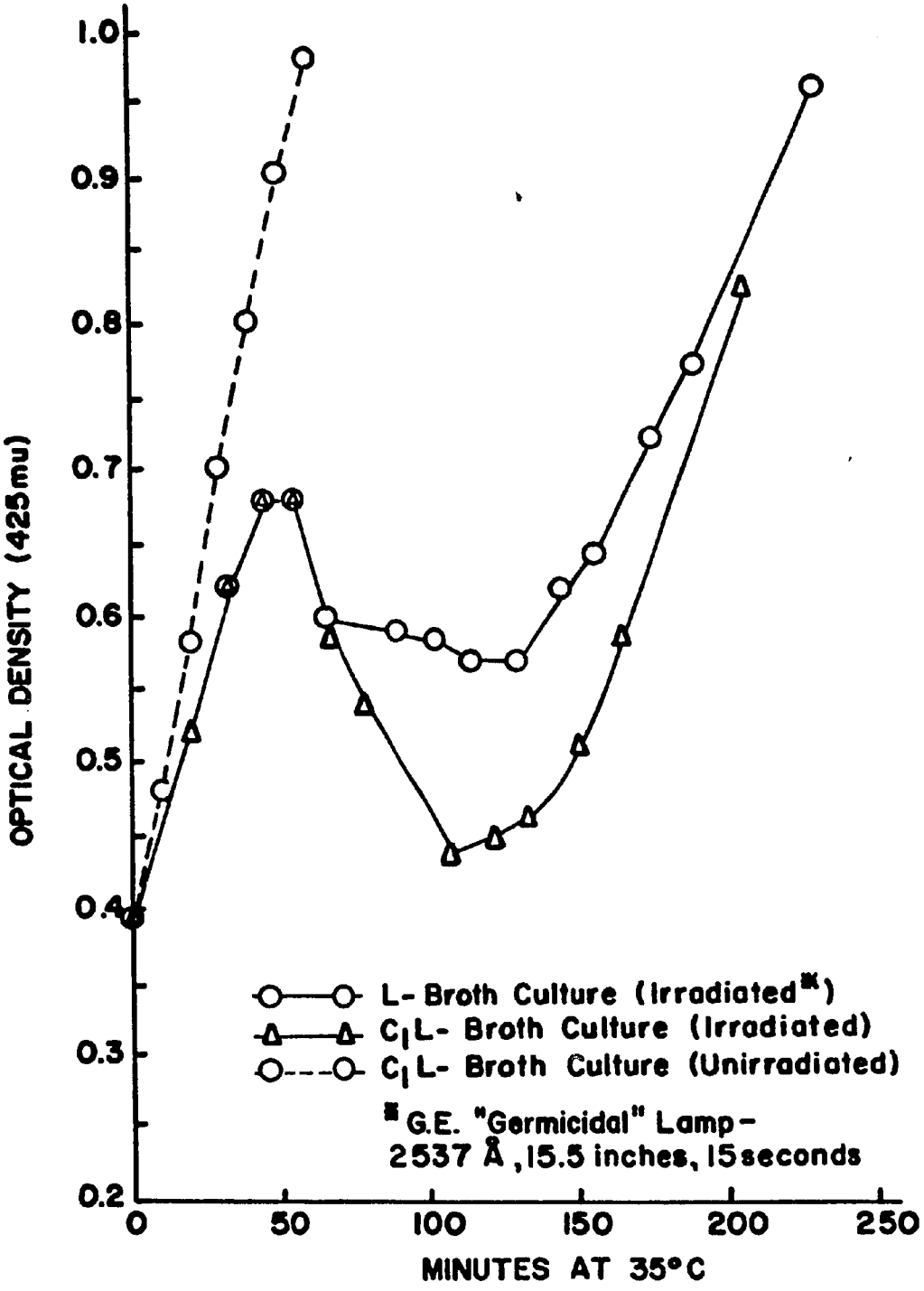


FiG. 4. Growth of Salmonella senftenberg 775W in citrated and non-citrated L-broth after ultraviolet irradiation.

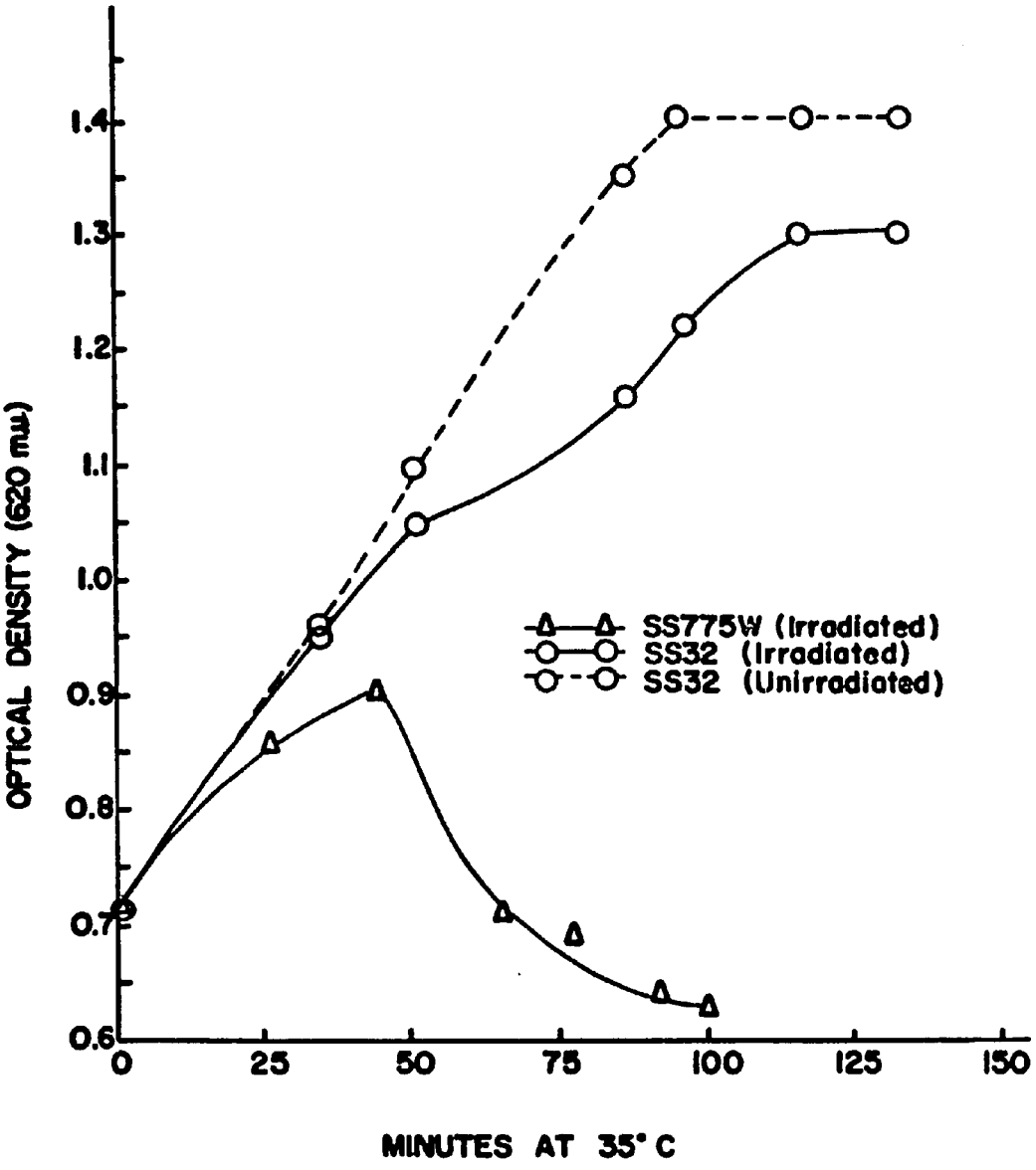


FiG. 5. Growth of Salmonella senftenberg strains 32 and 775W in citrated L-broth after ultraviolet irradiation.

employed for each individual experiment is described in detail in the "Materials and Methods" section of this presentation.

A list of all the isolates that were examined in this study for the characters of lysogeny and/or heat resistance is given in Table IX.

HRT Experiment No. 1

In the first HRT experiment a method of "cyclic" growth and thermal inactivation was used to select transductants (or convertants) with a transferred marker for heat resistance from mixed suspensions of phage and bacteria (PBM suspensions) and "normal" survivors from control (B) suspensions. In this experiment the progeny of the cells in the original suspensions were carried through three growth phases at 25°C and 35°C for at least 24 hours and two phases of heating at 60°C. Combined, these phases represent a total of 2 1/2 cycles of growth and thermal inactivation.

Thermal Inactivation Curves. Although their opinions on the mechanism of cellular death may differ, almost all workers agree that a decreased or decreasing slope in a survivor curve signifies a decrease in the rate of inactivation and indicates the presence of heat-resistant cells in a population. This decrease may appear in the concave, exponential, or convex portion of the curve. In this investigation, exponential rates of thermal inactivation were expressed as Decimal-Reduction Time (D_T) values. The D_T value indicates the time required for the inactivating temperature (T) to reduce the surviving portion of

a cell population by 90 percent leaving 10 percent survivors; it is also defined as the time required for a survivor curve to linearly traverse one log cycle. Increases in the D_T value for an organism under the same conditions of growth, heating, and recovery indicate that its rate of inactivation has decreased and that it has increased in heat resistance.

The thermal inactivation curves obtained for progeny of the original PBM and B cell suspensions heated at 60°C are shown in Figure 6. First and second cycle curves were plotted from survivor data obtained by diluting and heating one ml of a stationary phase culture of the 25B, 25PBM, 35B, and 35 PBM cells in 99 ml of broth medium preheated to 60°C. Plate counts of a 1:100 dilution of the unheated cultures were used to determine 100 percent survivors at "zero" time. Plate counts representing 100 percent survivors for the 25B, 25PBM, 35B and 35 PBM cultures during the first cycle of heating were 9.6×10^7, 6.6×10^7, 9.0×10^7, and 1.0×10^8 per ml. For the second cycle they were 1.1×10^8, 9.0×10^7, 1.0×10^8, and 1.1×10^8, respectively.

It can be seen from these curves that the cells exposed to SS775W phage (PBM cells) were more resistant to heating at 60°C than unexposed cells. In the heating phase of the first cycle only the PBM cells grown at 35°C (35 PBM cells) exhibited an increase in heat resistance. This was evidenced by the slower rate at which these cells were inactivated during the first 2.5 minutes of heating in comparison with the control cells and by the decreasing rate change noted for these cells

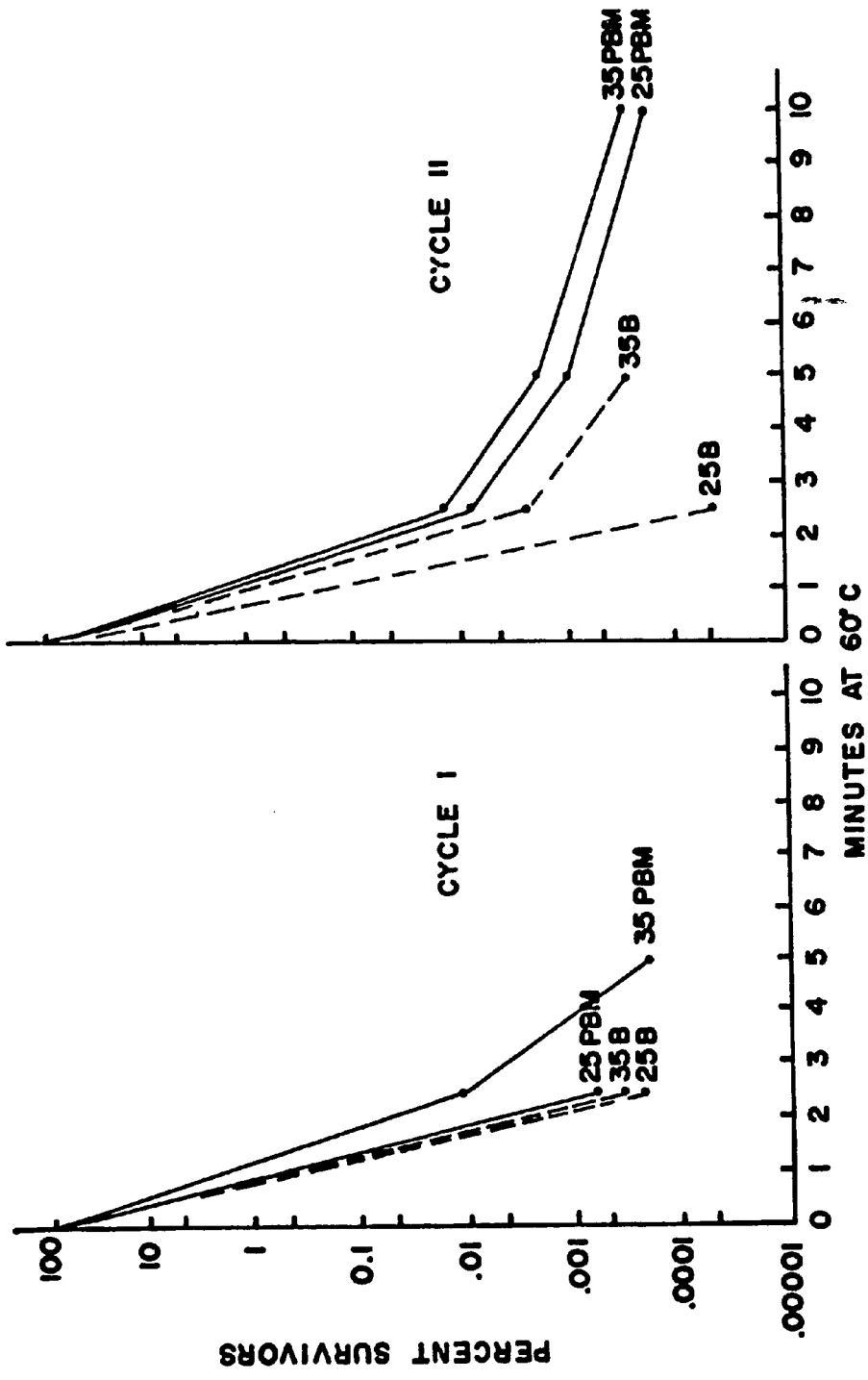


FiG. 6. Thermal inactivation curves for S. senftenberg strain 32 after exposure to S. senftenberg bacteriophage PSS775W in HRT experiment No. 1.

at the 0.01 percent survivor level. In the second cycle, the inactivation rate for the 35 PBM cells remained the same for the same period of time but the decreasing change in rate at the 0.01 percent survivor level was significantly greater. Similar death rates for the 35 PBM curves in the initial stages of heating in both cycles would not have occurred if the decreasing death rate noted in the first cycle was due to a selection of cells that had increased in heat resistance and had become the predominant cells in the population inactivated in the second cycle. In all probability, this decrease reflects the presence of the older cells present in the stationary-phase cell population which are inherently more thermal-resistant. The increased heat resistance of the 25 PBM cells, although not expressed until the second cycle, appears to be comparable to that of the 35 PBM cells. In the same cycle, an increase in heat resistance was observed for the 35 B cells but not for the 25 B cells.

The D_{60} values calculated for the 35 PBM, 25 PBM, 35 B and 25 B cells during the first 2.5 minutes of heating at 60°C were 0.64, 0.49, 0.46, and 0.45 minutes for the first cycle and 0.66, 0.62, 0.55, and 0.40 minutes for the second cycle, respectively. The D_{60} values calculated for the time intervals in minutes (enclosed in parenthesis) following this initial time period were 1.41 (2.5 - 5.0) for 35 PBM (cycle I); 2.84 (2.5 - 5.0) and 6.10 (5.0 - 10.0) for 35 PBM (cycle II); 2.76 (2.5 - 5.0) and 6.67 (5.0 - 10.0) for 25 PBM (cycle II); and 2.57 (2.5 - 5.0) for 35 B (cycle II).

Identification of Isolates. In all, 240 "PBM" isolates and 131 "B" isolates were recovered in this experiment (see Tables IV, V, VI, VII). The isolates were alphabetically grouped (A to D) on the basis of their cycle of recovery (I, II) and growth temperature (25°C, 35°C) and were given a subscript number to designate them as PBM isolates (A_1 to D_1) or B isolates (A_2 to D_2).

Each of the isolates was identified by an "isolate" number (PBM1 - PBM240, B1 - B131) and a "treatment and recovery identification" number (TRI number) which is a combination of (a) "sample" number-cycle of recovery (I,II); processing temperature (60°C); growth temperature (25°C, 35°C); and processing time in minutes; and (b) the "count-dilution-colony" number (CDC number) - total count/volume of the sample dilution plated; the dilution factor (En = 10^n); and the colony number.

Tests for Lysogeny and Thermal Resistance. Of the 240 "PBM" isolates and 131 "B" isolates recovered in this experiment only 17 of the PBM isolates were tested for the characters of lysogeny and increased heat resistance (see Table X). The following notes are given to clarify some of the column headings and entries in this Table.

Column I. These isolates are identified separately in Table IX.

Column II. The "spinner" bottle method was used to screen the isolates for heat resistance. Survivors were determined as "pour plate" counts in L-Agar (35°C, 72 hours).

TABLE IV

IDENTIFICATION OF ISOLATES RECOVERED IN HEAT RESISTANCE TRANSDUCTION EXPERIMENT NO. 1 (GROUP NOS. A1 and B1)

Isolate No.	Group No.	TRI No.[a]	
		Sample No.	CDC No.[b]
PBM1 -PBM6	A1	I-60/35-0	100E7-(1 to 6)
PBM7 -PBM8	"	" -2.5	2E3 -(1 to 2)
PBM9 -PBM11	"	" -2.5	18E2 -(1 to 3)
PBM12 -PBM14	"	" -5	3E1 -(1 to 3)
PBM15 -PBM36	"	" -5	22E0 -(1 to 22)
PBM37	"	" -7.5	1E1 -1
PBM38 -PBM42	"	" -7.5	5E0 -(1 to 5)
PBM43 -PBM49	"	" -15	22E1 -(1 to 7)
PBM50 -PBM53	"	" -20	4E1 -(1 to 4)
PBM54 -PBM60	"	" -30	7E0 -(1 to 7)
PBM61 -PBM65	"	" -45	6E0 -(1 to 5)
PBM66 -PBM70	"	" -60	5E0 -(1 to 5)
PBM71 -PBM75	"	" -80	5E0 -(1 to 5)
PBM76 -PBM81	B1	I-60/25-0	6E8 -(1 to 6)
PBM82 -PBM84	"	" -2.5	3E1 -(1 to 3)
PBM85 -PBM94	"	" -2.5	42E0 -(1 to 10)
PBM95	"	" -5	1E0 -1
PBM96 -PBM97	"	" -7.5	2E1 -(1 to 2)
PBM98 -PBM106	"	" -7.5	9E0 -(1 to 9)
PBM107	"	" -10	1E0 -1
PBM108	"	" -40	1E0 -1
PBM109	"	" -45	1E0 -1

[a] Treatment and Recovery Identification Number

[b] "Count-Dilution-Colony" Number. Counts are given per volume of suspension plated which differs for some of the groups of isolates. For Groups A1 and B1 the volume plated was 0.1 ml.

TABLE V

IDENTIFICATION OF ISOLATES RECOVERED IN HEAT RESISTANCE TRANSDUCTION EXPERIMENT NO. 1 (GROUP NOS. C1 and D1)

Isolate No.	Group No.	TRI No.[a]	
		Sample No.	CDC No.[b]
PBM110-PBM115	C1	II-60/35-0	14E8 -(1 to 6)
PBM116-PBM121	"	" -2.5	19E2 -(1 to 6)
PBM122-PBM125	"	" -5	4E2 -(1 to 4)
PBM126-PBM129	"	" -10	4E1 -(1 to 4)
PBM130	"	" -15	1E1 -1
PBM131-PBM132	"	" -15	2E0 -(1 to 2)
PBM133-PBM138	"	" -30	6E0 -(1 to 6)
PBM139	"	" -40	1E0 -1
PBM140	"	" -50	1E0 -1
PBM141-PBM152	"	" -60	12E0 -(1 to 12)
PBM153-PBM156	"	" -70	4E0 -(1 to 4)
PBM157-PBM170	"	" -90	14E0 -(1 to 14)
PBM171-PBM176	D1	II-60/25-0	9E0 -(1 to 6)
PBM177-PBM185	"	" -2.5	9E2 -(1 to 9)
PBM186-PBM195	"	" -2.5	78E1 -(1 to 10)
PBM196	"	" -5	1E2 -1
PBM197-PBM204	"	" -5	8E1 -(1 to 8)
PBM205-PBM210	"	" -5	106E0-(1 to 6)
PBM211-PBM227	"	" -10	17E0 -(1 to 17)
PBM228-PBM235	"	" -15	8E0 -(1 to 8)
PBM236-PBM237	"	" -20	2E0 -(1 to 2)
PBM238	"	" -25	1E0 -1
PBM239-PBM240	"	" -30	2E0 -(1 to 2)

[a] Treatment and Recovery Identification Number

[b] "Count-Dilution-Colony" Number. Counts are given per volume of suspension plated which differs for some of the groups of isolates. For Groups C1 and D1 the volume plated was 0.1 ml.

TABLE VI

IDENTIFICATION OF ISOLATES RECOVERED IN HEAT RESISTANCE TRANSDUCTION EXPERIMENT NO. 1 (GROUP NOS. A2 and B2)

Isolate No.	Group No.	TRI No.[a]	
		Sample No.	CDC No.[b]
B1 - B6	A2	I-60/35-0	100E7-(1 to 6)
B7 - B12	"	" -2.5	6E1 -(1 to 6)
B13	"	" -5	1E1 -1
B14 - B20	"	" -5	7E0 -(1 to 7)
B21 - B23	"	" -15	3E1 -(1 to 3)
B24 - B37	"	" -15	15E0 -(1 to 14)
B38	"	" -20	1E0 -1
B39 - B42	"	" -35	4E0 -(1 to 4)
B43	"	" -60	1E0 -1
B44 - B49	B2	I-60/25-0	12E8 -(1 to 6)
B50 - B51	"	" -5	2E1 -(1 to 2)
B52 - B61	"	" -5	24E0 -(1 to 10)
B62	"	" -10	1E0 -1
B63 - B64	"	" -15	2E0 -(1 to 2)
B65 - B66	"	" -20	2E0 -(1 to 2)
B67	"	" -25	1E0 -1
B68 - B72	"	" -30	5E0 -(1 to 5)
B73	"	" -35	1E0 -1
B74 - B76	"	" -40	3E0 -(1 to 3)

[a] Treatment and Recovery Identification Number

[b] "Count-Dilution-Colony" Number. Counts are given for volume of suspension plated which differs for some of the groups of isolates. For Groups A2 and B2 the volume plated was 0.1 ml.

TABLE VII

IDENTIFICATION OF ISOLATES RECOVERED IN HEAT RESISTANCE TRANSDUCTION EXPERIMENT NO. 1 (GROUP NOS. C2 and D2)

Isolate No.	Group No.	TRI No. [a]	
		Sample No.	CDC No.[b]
B77 - B81	C2	II-60/35-0	5E8 -(1 to 5)
B82 - B84	"	" -2.5	3E2 -(1 to 3)
B85 - B90	"	" -2.5	23E1 -(1 to 6)
B91	"	" -5	1E2 -1
B92 - B95	"	" -5	4E1 -(1 to 4)
B96 - B99	"	II-60/35-10	4E0 -(1 to 4)
B100 - B101	"	" -15	2E0 -(1 to 2)
B102 - B107	"	" -20	35E1 -(1 to 6)
B108 - B110	"	" -30	3E0 -(1 to 3)
B111	"	" -40	1E0 -1
B112 - B117	"	" -70	23E0 -(1 to 6)
B118 - B119	"	" -90	2E0 -(1 to 2)
B120 - B125	D2	II-60/25-0	14E8 -(1 to 6)
B126 - B130	"	" -2.5	5E0 -(1 to 5)
B131	"	" -5	1E0 -1

[a] Treatment and Recovery Identification Number

[b] "Count-Dilution-Colony" Number. Counts are given for volume of suspension plated which differs for some of the groups of isolates. For Groups C2 and D2 the volume plated was 0.1 ml.

TABLE VIII

IDENTIFICATION OF ISOLATES RECOVERED IN HEAT RESISTANCE TRANSDUCTION EXPERIMENTS NO. 2 and NO. 4

Isolate No.	Group No.	TRI No. [a]	
		Sample No.	CDC No.[b]
EXPERIMENT HRT-2			
PBM250 - PBM258	E1	I-55/35-2	9E5 -(1 to 9)
PBM259 - PBM265	"	" -4	7E3 -(1 to 7)
PBM266 - PBM273	"	" -5	12E2-(1 to 8)
PBM274 - PBM301	F1	II-55/35-60	46E0-(1 to 28)
PBM302 - PBM321	G1	III-55/35-15	134E1-(1 to 20)
B150 - B158	E2	I-55/35-2	9E2 -(1 to 9)
B159 - B165	"	" -4	7E1 -(1 to 7)
B166 - B176	"	" -5	11E1 -(1 to 11)
B177 - B180	F2	II-55/35-60	4E0 -(1 to 4)
B181 - B184	"	" -50	4E0 -(1 to 4)
B185 - B189	"	" -40	6E0 -(1 to 5)
B190 - B194	G2	III-55/35-15	7E0 -(1 to 5)
EXPERIMENT HRT-4			
PBM400 - PBM418	H1	I-60/35-3	19E0 -(1 to 19)
B400 - B402	H2	I-60/35-3	3E0 -(1 to 3)

[a] Treatment and Recovery Identification Number

[b] "Count-Dilution-Colony" Number. Counts are given for volume of suspension plated which differs for some of the groups of isolates. Group G is given in count per 0.1 ml, Groups E and F in count per 1.0 ml and Group H in count per 2.0 ml.

TABLE IX

IDENTIFICATION OF ISOLATES RECOVERED IN HEAT RESISTANCE TRANSDUCTION EXPERIMENTS AND EXAMINED FOR THE CHARACTERS OF LYSOGENY AND/OR HEAT RESISTANCE

Isolate No.	Group No.	TRI No.[a]	
		Sample No.	CDC No.[b]
HRT-1 Isolates			
PBM 54 - PBM 60	A1	I-60/35-30	7E0-(1 to 7)
PBM 108	B1	I-60/25-40	1E0-1
PBM 109	B1	I-60/25-45	1E0-1
PBM 133 - PBM 138	C1	II-60/35-30	6E0-(1 to 6)
PBM 239 - PBM 240	D1	II-60/25-30	2E0-(1 to 2)
HRT-2 Isolates			
PBM 250 - PBM 258	E1	I-55/35- 2	9E5-(1 to 9)
PBM 259 - PBM 265	E1	I-55/35- 4	7E3-(1 to 7)
PBM 266 - PBM 273	E1	I-55/35- 5	12E2-(1 to 8)
PBM 274 - PBM 301	F1	II-55/35-60	46E0-(1 to 28)
B 178	F2	II-55/35-60	4E0-1
B 183 - B 184	F2	II-55/35-50	4E0-(3,4)
PBM 302 - PBM 321	G1	III-55/35-15	134E1-(1 to 20)
B 190 - B 194	G2	III-55/35-15	7E0-(1 to 5)
HRT-4 Isolates			
PBM 400 - PBM 418	H1	I-60/35- 3	19E0-(1 to 19)
B 400 - B 402	H2	I-60/35- 3	3E0-(1 to 3)

[a] Treatment and Recovery Identification Number

[b] "Count-Dilution-Colony" Number. Counts are given per volume of suspension plated which differs for the various isolate groups. Groups A - D and Group G (Counts/0.1 ml); Groups E and F (Counts/ml); and Group H (Counts/2 ml).

TABLE X

TESTS FOR LYSOGENY AND INCREASED HEAT RESISTANCE ON EXPERIMENT HRT-1 "FIRST AND SECOND CYCLE" ISOLATES

Group and Isolate Numbers	Screening Test for Increase in Thermal Resistance		Spot/Lawn Tests for Phage Infectivity and Immunity			
	% Survivors (60°C,2.5 min.)	Relative Increase in % S	Isolate / SS32	PSS775W / Isolate	Isolate / SS775W	SS32 / Isolate
A1						
PBM 54	9.0×10^{-4}	204	-	+	-	-
PBM 55	1.5×10^{-6}	-0.6	-	+	-	-
PBM 56	1.1×10^{-5}	1.5	-	+	-	-
PBM 57	1.5×10^{-6}	-0.6	-	+	-	-
PBM 58	2.9×10^{-6}	-0.3	-	+	-	-
PBM 59	3.6×10^{-6}	-0.2	-	+	-	-
PBM 60	5.8×10^{-6}	0.3	-	+	-	-
B1						
PBM 108	2.4×10^{-4}	54	+	-	-	-
PBM 109	7.3×10^{-7}	-0.8	+	-	-	-
C1						
PBM 133	2.4×10^{-3}	544	-	+	-	-
PBM 134	1.5×10^{-3}	340	-	+	-	-
PBM 135	7.3×10^{-7}	-0.8	+	-	-	-
PBM 136	$< 7.3 \times 10^{-7}$	(-0.8)	-	+	-	-
PBM 137	7.3×10^{-7}	-0.8	-	+	-	-
PBM 138	1.3×10^{-2}	2954	+	-	-	-
D1						
PBM 239	3.8×10^{-4}	85	-	+	-	-
PBM 240	7.3×10^{-7}	-0.8	-	+	-	-
Controls						
SS32	4.4×10^{-6} (4.4×10^{-6})	0	-	+	-	-
SS775W	77.4×10^{0} (84.1×10^{0})	(17.6×10^{6})	+	-	-	-

+ = lysis of lawn culture

Percent survivor (%S) values for both the isolates and the donor (SS775W) and recipient (SS32) strains are based on 100% = 1.37×10^8 viable cells/ml which is an average of the values obtained for the donor (1.26×10^8 cells/ml) and the recipient (1.46×10^8 cells/ml). Percent survivor values for SS775W and SS32 based on the actual values obtained appear in the table in parenthesis.

Optical Density (O.D.) readings at 425 mμ were taken of each 24-hour "isolate" culture (1/100 dilution) used in this test but no attempt was made to adjust the culture to give a final concentration of 1×10^8 in the suspending medium. For PBM 54-PBM60, the O.D. readings were: .114, .119, .137, .125, .119, .125, and .131; for PBM108 and PBM109: .114 and .119; for PBM133 to PBM138: .125, .114, .102, .108, and .125; for PBM239 and PBM 240: .125 and .125; and for SS32 and SS775W: .125 and .125 respectively. An O.D. reading of 0.1 represents 1×10^8 viable cells/ml.

Column III. Increase in thermal resistance was measured as the "relative increase in %S" which is defined here as the "ratio of the difference in %S values obtained for the isolate (I) and the recipient (R) strain to that of the recipient" and is calculated from the expression $\frac{\%SI - \%SR}{\%SR}$. Using this expression, values indicating an increase (or decrease)

in thermal resistance can be obtained which are dependent on the conditions employed for the screening test. A value of 0 indicates no increase in percent survivors and values of 1 and -1 indicate, respectively, a one-fold (100%) increase and decrease.

Columns IV, V, VI, and VII. Spot tests (spot/lawn tests) for phage susceptibility and immunity of the isolates and control strains were performed by placing a drop or loopful of the material to be tested onto previously prepared lawn cultures and observing for evidence of lysis of the lawn after a suitable incubation period (six hours at 35°C). Lawn lysis is indicated by a plus (+) sign. Each lawn culture was prepared as a circular patch using a loopful of a "young" L-Broth culture containing ca, $1x10^7$ CFU/ml. It should be noted that the column headings under discussion are separated by a slanted line to indicate the spotted material (upper heading) and the lawn cultures on which it was placed (lower heading).

All of the 17 PBM isolates recovered in the first HRT experiment and examined for the properties of lysogeny (ly^+) and heat resistance (hr^+) represent survivors in populations that have been reduced to a level of less than 0.0001 percent by heating at 60°C for between 30 to 45 minutes. Nine of the

isolates tested were from the first cycle and eight were from the second cycle (see Tables X and IX, and Figure 6). Of the nine "first cycle" isolates, seven (PBM 54 - PBM 60) were 35 PBM isolates recovered after 30-minute heating at 60°C and the remaining two were 25 PBM isolates (PBM 108 and PBM 109) recovered after 40 and 45 minutes, respectively. All of the eight "second cycle" PBM isolates were obtained after 30 minutes of heating at 60°C. Six of these (PBM 133 - PBM 138) were 35 PBM isolates and the remainder (PBM 239 and PBM 240) were 25 PBM isolates.

Tests for phage infectivity and immunity (columns IV to VII, Table X) indicated that all the "first cycle" 35 PBM isolates and "second cycle" 25 PBM isolates were nonlysogenic (ly^-) and all the "first cycle" 25 PBM isolates were lysogenic (ly^+). Of the six 35 PBM isolates recovered in the second cycle, two were found to be lysogenic (PBM 135 and PBM 138) and the other four to be nonlysogenic.

The results of the screening tests employed to detect an increase in thermal resistance (columns II and III, Table X) indicate that only six of the 17 isolates (PBM54, -108, -133, -134, -138, and -239) have RIPS values of sufficient magnitude (50 to 3,000) to warrant consideration as "hr" convertants or transductants. The remaining isolates had values ranging from less than -0.8 to 1.5 and two of these carried SS775W prophage. These values, however, represent only a fraction of the RIPS value of 17,600,000 calculated for SS775W.

The heat resistance and lysogenic properties (hr, ly), calculated RIPS values, and sample numbers of the above isolates suspected as being convertants or transductants are as follows:

PBM 54: hr^+, ly^-, RIPS = 204, I-60/35-30;

PBM 108: hr^+, ly^+, RIPS = 54, I-60/35-40;

PBM 133: hr^+, ly^-, RIPS = 544, II-60/35-30;

PBM 134: hr^+, ly^-, RIPS = 340, II-60/35-30;

PBM 138: hr^+, ly^+, RIPS = 2954, II-60/35-30; and

PBM 239: hr^+, ly^-, RIPS = 85, II-60/25-30.

HRT Experiment No. 2

In this experiment, the progeny of the cells in the test (PBM) suspension and the control (B) suspension were carried through three cycles of growth at 35°C and heating at 55°C in an attempt to select "hr" transductants or convertants.

Growth and Thermal Inactivation Curves. Turbidimetric growth curves were plotted for "PBM" cultures only during the first two cycles of growth and heating (Figure 7). The third growth phase was carried for 26 hours to stationary phase at which time samples were removed for heating. The ".01 sample" cultures (i.e., overnight cultures containing approximately 0.01% survivors) used in the second phase of growth were from late-log phase cultures of "B" cells heated in the first cycle for two minutes to give 0.011 percent survivors and of "PBM" cells heated for four minutes to give 0.032 percent survivors. In the third phase of growth, the ".01 sample" cultures were

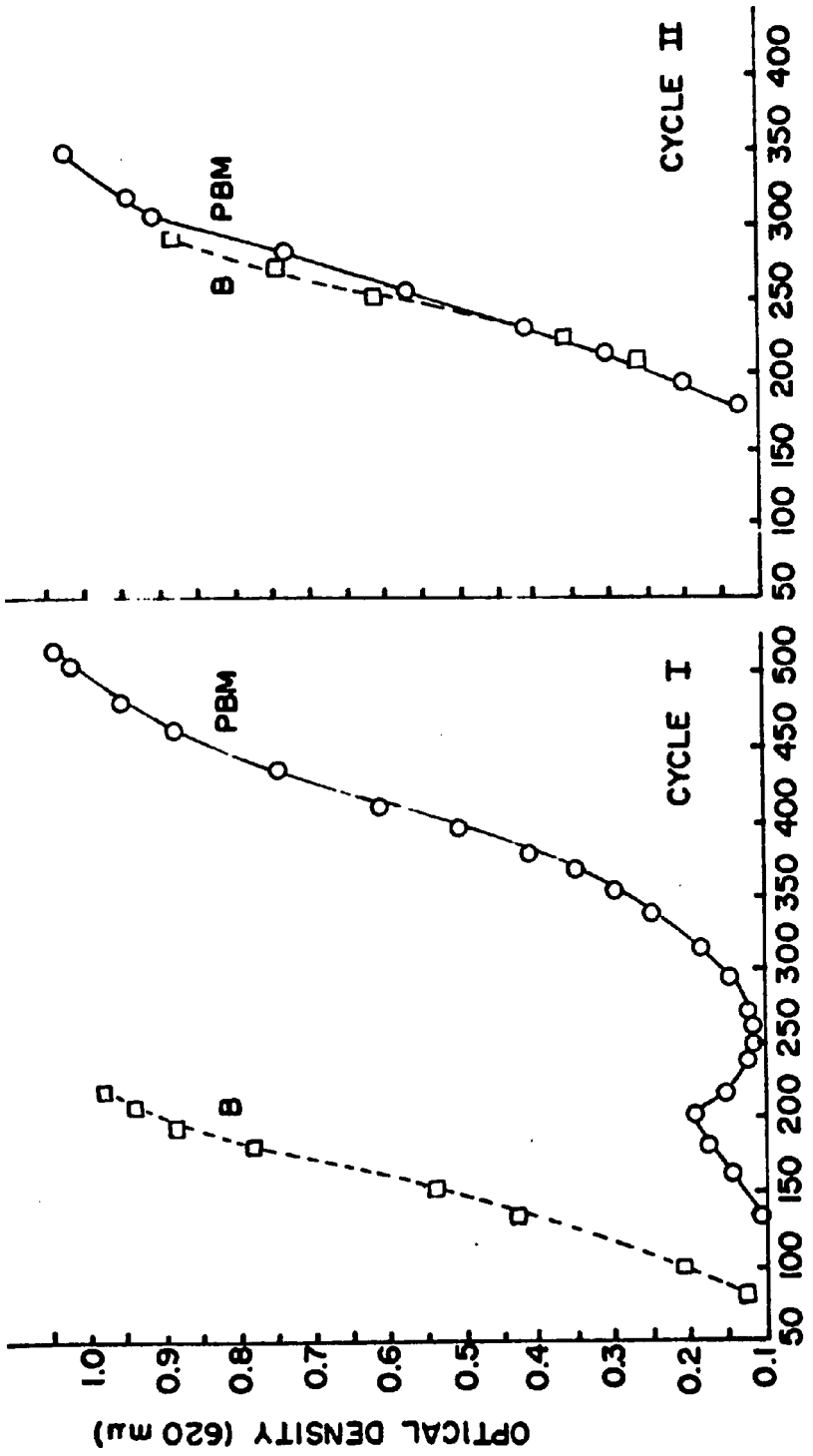


FIG. 7. Growth curves for S. senftenberg strain 32 after exposure to S. senftenberg bacteriophage PSS775W in HRT experiment No. 2.

from late-log phase cultures of "B" cells heated in the second cycle for 60 minutes (0.00036% survivors) and from "PBM" cells heated for the same period of time (0.00042% survivors).

Thermal inactivation curves were plotted for samples of "B" and "PBM" cultures removed at various stages during the growth phases of the three cycles and subjected to heating at 55°C (Figure 8). For the first two cycles the cells were in the late-log phase of growth (ca, $1x10^9$/ml) and for the third cycle they were in the stationary phase of growth. Plate counts representing 100 percent survivors at "zero" time for cultures of the "B" cells and "PBM" cells were $9.6x10^6$ and $6.8x10^6$ for the first cycle; $1.1x10^7$ and $1.1x10^7$ for the second cycle; and $3.1x10^6$ and $3.0x10^6$ for the third cycle.

The thermal inactivation curve for the second "growth and heating" cycle shown in Figure 8 was only plotted for a 20-minute heating period. Actually, survivors were still being recovered at sampling times of 25, 30, 40, 50, and 60 minutes. For the "B" cultures the colony counts for these times were 10, 13, 6, 4, and 4 per ml of sample; for the "PBM" cultures they were 64, 43,29, 29, and 46 per ml of sample, respectively.

Low levels of survivors were also recovered from samples of "first cycle" cultures heated for 7.5, 10, 15, 20, and 30 minutes, which amounted to less than 4 per 0.1 ml for the "B" culture and less than 9 per 0.1 ml for the "PBM" culture, but were not recovered (<1/0.1 ml) from the samples of "third cycle" cultures heated for 60 minutes.

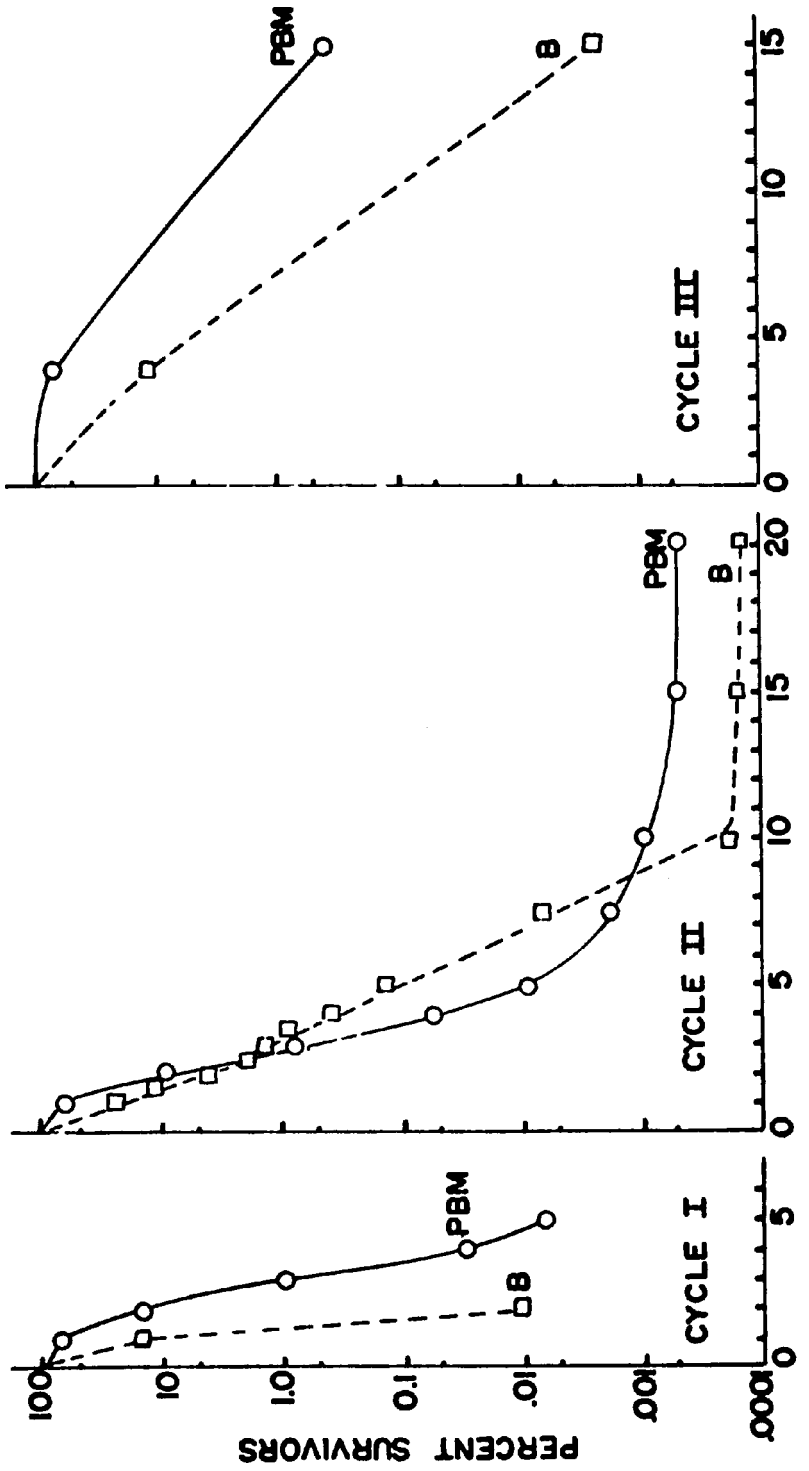


FiG. 8. Thermal inactivation curves for S. senftenberg strain 32 after exposure to S. senftenberg bacteriophage PSS775W in HRT experiment No. 2.

For the time intervals in minutes indicated in parenthesis the respective D_{55} values calculated for the surviving cell population in the heating phases of cycles I, II and III were 0.74 (2.4 - 4.0), 0.74 (1.0 - 4.0), and 5.0 (4.0 - 15.0) for the PBM cells, and 0.31 (1.0 - 2.0), 1.34 (0.8 - 2.0), and 1.93 (2.0 - 10.0) and 2.97 (3.0 - 15.0) for the B cells.

The increased heat resistance of the PBM cells over the control (B) cells can be seen in the thermal inactivation curves plotted for all three "growth and heating" cycles in Figure 8. Compared with the inactivation curves for the control cells, the curves for the PBM cells exhibited more pronounced "shouldering" in all cycles, had lower inactivation rates in cycles I and III, and in cycle II displayed more persistent "tailing" at the higher survivor level of 0.0004 percent. The rate of inactivation of the PBM cells was the same in cycles I and II (D_{55} = 0.74 minutes) but lower in cycle III (D_{55} = 5.0 minutes).

Identification of Isolates. In the second transduction experiment, 72 "PBM" isolates and 45 "B" isolates were recovered (see Table VIII). These isolates were grouped (E to G) on the basis of their cycle of recovery (I, II, III) and assigned subscript numbers to designate them as "PBM" isolates (E_1 to G_1) or as "B" isolates (E_2 to G_2). As in the first HRT experiment, each isolate was identified by an "isolate" number (PBM250-PBM321, B150-B194) and a "treatment and recovery identification" number.

Tests for Lysogeny and Thermal Resistance. Tests for lysogeny and/or heat resistance were performed on all 72 of the "PBM" isolates recovered in this experiment and on 8 of the 45 "B" isolates. These isolates are identified in Table IX. Twenty-four of the "PBM" isolates were recovered in the first cycle, 28 in the second cycle, and 20 in the third. Of the 8 "B" isolates, 3 were from the second cycle and the remainder from the third.

Screening tests to detect survivors with increased heat resistance were carried out with 24-hour cultures of the isolates; the heat-resistant, lysogenic, donor strain (SS775W); and the heat-sensitive, nonlysogenic, recipient strain (SS32). For these tests, the cultures were diluted 10^{-1} or 10^{-2} in broth and heated at 60°C for 2.5 minutes. The relative heat resistance of the isolates was determined by comparing either the numbers of survivors or the colony development patterns (see Table XIV) obtained for the isolates with those obtained for positive (SS75W) and negative (SS32) controls. Numbers of survivors were determined by the "plate count" method and colony development patterns by an agar "spot" plate method.

Twenty-five "third cycle" isolates were screened for thermal resistance using the "plate count" method (Tables XI, XII and XIII). Of this total, 20 were "PBM" isolates and 5 were "B" isolates. These isolates represented survivors from populations that had been reduced to a level of 0.42 percent (PBM isolates) or 0.0023 percent (B isolates) by heating at 55°C in broth for a period of 15 minutes (see Cycle III,

TABLE XI

TESTS FOR INCREASED HEAT RESISTANCE ON EXPERIMENT HRT-2 "THIRD CYCLE" ISOLATES (TEST SERIES NO. 1)

Isolate No.	Turbidity of Test Cell Suspension at 10^{-1} Dilution[a] ($\%T_{425}$)	No. of Survivors Per Ml of Heated Cell Suspension[b] (L-Agar: 35°C, 48 hrs)
PBM 302	81	1.4×10^6
PBM 303	78	3.6×10^6
PBM 304	76.5	2.1×10^6
PBM 305	79	8.2×10^4
PBM 306	80	3.1×10^5
PBM 307	79.5	2.6×10^6
PBM 308	78	3.5×10^5
PBM 309	78.5	4.1×10^5
PBM 310	79	5.7×10^5
PBM 311	79	2.6×10^5
SS 32	81	1.1×10^5
SS 775W	78.5	1.3×10^9

[a] Test cell suspensions were prepared in L-Broth at a concentration of approximately 1×10^9 cells per ml (10^0 dilution) from 24 hr (35°C), broth shaker cultures; $\%T_{425}$ = Percent Transmittance at 425 mu.

[b] The suspensions were heated at 60°C for 2.5 minutes in sealed, one ml, serological pipettes. Survivors were determined from L-Agar "pour plate" counts

TABLE XII

TESTS FOR INCREASED HEAT RESISTANCE ON EXPERIMENT HRT-2 "THIRD CYCLE" ISOLATES (TEST SERIES NO. 2)

Isolate No.	Turbidity of Test Cell Suspension at 10^{-1} Dilution[a] ($\%T_{425}$)	No. of Survivors Per Ml of Heated Cell Suspension[b] (L-Agar: 35°C, 72 hrs)
PBM 312	79	4.1×10^6
PBM 313	79	3.2×10^6
PBM 314	78	2.9×10^6
PBM 315	78.5	2.4×10^6
PBM 316	79.5	4.4×10^6
PBM 317	79.5	3.3×10^6
PBM 318	80	5.3×10^6
PBM 319	78.5	5.0×10^6
PBM 320	77.5	3.1×10^6
PBM 321	79	3.0×10^6
SS 32	80	7.6×10^5
SS 775W	78.5	9.2×10^8

[a] Test cell suspensions were prepared in L-Broth at a concentration of approximately 1×10^9 cells per ml (10^0 dilution) from 24 hr (35°C), broth shaker cultures; $\%T_{425}$ = Percent Transmittance at 425 mu.

[b] The suspensions were heated at 60°C for 2.5 minutes in sealed, one ml, serological pipettes. Survivors were determined from L-Agar "pour plate" counts.

TABLE XIII

TESTS FOR INCREASED HEAT RESISTANCE ON EXPERIMENT HRT-2 "THIRD CYCLE" ISOLATES (TEST SERIES NO. 3)

Isolate No.	Turbidity of Test Cell Suspension at 10^{-1} Dilution[a] ($\%T_{425}$)	No. of Survivors Per Ml of Heated Cell Suspension[b] (L-Agar: 35°C)	
		24 hours	5 days
PBM 303	79.5	1.5×10^6	8.9×10^6
PBM 308	79.5	4.7×10^5	4.3×10^6
B 190	81	7.3×10^5	3.1×10^6
B 191	81.5	1.2×10^6	3.6×10^6
B 192	81.5	1.0×10^5	9.8×10^5
B 193	80.5	5.6×10^5	2.4×10^6
B 194	83.0	9.4×10^4	6.6×10^5
SS 32	80.5	1.0×10^5	9.6×10^5
SS 775W	78.5	4.8×10^8	4.9×10^8

[a] Test cell suspensions were prepared in L-Broth at a concentration of approximately 1×10^9 cells per ml (10^0 dilution) from 24 hr (35°C), broth shaker cultures; $\%T_{425}$ = Percent Transmittance at 425 mu.

[b] The suspensions were heated at 60°C for 2.5 minutes in sealed, one ml, serological pipettes. Survivors were determined from L-Agar "pour plate" counts.

Figure 8). The tests were performed in three series, each with its own positive (SS75W) and negative (SS32) controls. All tests suspensions were heated by the "sero-pipette" method and contained ca, $1x10^9$ cells per ml. Survivors were determined as "pour" plate counts on L-Agar at 35°C but at different time intervals of incubation. For series No. 1, 2 and 3, the survivors counts represent recovery times of 48 hours, 72 hours, and 24 hours and 5 days, respectively.

The concentration of cells in the unheated test suspensions were estimated from percent transmittance readings at 425 mμ ($\%T_{425}$) obtained for 10^{-1} dilutions of the test suspensions in broth. For stationary phase cultures of salmonellae diluted 10^{-2} in broth, an 80 $\%T_{425}$ reading was found to be approximately equal to $1x10^8$ CFU per ml.

First and second cycle isolates were tested for increased heat resistance by a "spot" plate method (Table XV). From a total of 52 "PBM" isolates tested by this method, 24 were recovered in the first cycle (PBM250 - PBM237) and 28 in the second (PBM274 - PBM301). All test suspensions were heated at 60°C for 2.5 minutes by the "capillary pipette" method and contained ca, $1x10^8$ cells per ml. Survivors were determined as colony development patterns on L-Agar and BGA medium following incubation at 35°C for 24 hours. For comparison with positive and negative controls, the isolates were grouped according to the similarity of their colony development patterns on BGA and L-Agar medium.

Representative colony development patterns for heated and unheated cell-broth suspensions of SS775W and SS32 on BGA medium after 24 hours at 35°C are shown schematically in Table XIV. The cell-broth suspensions used for this test were prepared from 24-hour - 35°C, broth cultures and contained $1x10^9$ viable cells per ml. The test suspensions were heated at 60°C for 2.5 minutes by the "sero-pipette" method and dilutions of the heated suspensions were "spotted" on the recovery medium with a loop. Following incubation, the areas spotted with the dilutions of the heated suspensions were observed for the presence or absence of growth and the appearance of the colonies that had developed in the inoculated areas. The type of colony development patterns seen at each dilution was recorded according to the following scheme: confluent growth (4+); semi-confluent (3+); 10 or more isolated colonies (2+); less than 10 isolated colonies (1+); and no growth (0). Isolates recovered from the second cycle have been employed in a study to determine the effect of incubation time and temperature on colony development patterns of S. senftenberg strains on BGA medium (Table XVI).

Tests to determine if the "PBM" isolates recovered in this experiment carried lysogens for SS775W were performed on 20 "PBM" isolates (PBM302 - PBM321) and 5 control "B" isolates (B191 - B194) using the qualitative assay test for phage described in the "Materials and Methods" section. All of these isolates were recovered in the third cycle of growth and

TABLE XIV

DETERMINATION OF HEAT RESISTANCE IN STRAINS OF SALMONELLA SENFTENBERG USING AN AGAR "SPOT" PLATE METHOD TO DETECT SURVIVORS IN DILUTIONS OF HEATED CELL SUSPENSIONS[a]

Strains	Colony Development Patterns on BGA Recovery Medium (24 hrs, 35C)							
	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
SS775W (unheated)	4+	4+	4+	4+	3+	2+	1+	0
SS775W (heated)	4+	4+	4+	4+	3+	2+	1+	0
SS32 (unheated)	4+	4+	4+	4+	3+	2+	1+	0
SS32 (heated)	4+	3+	2+	1+	0	0	0	0

[a]Cells from 24 hr. (35^{o}C) broth cultures were suspended in L-Broth (10^{0} dilution = 1×10^{9} cells/ml) and heated at 60^{o}C for 2.5 minutes in 1 ml pipettes sealed at the tip. Dilutions of the heated suspensions were "spotted" on the recovery medium with a 3mm wire loop that transferred a volume of ca 1/230 ml.

[b]Numerical Key: Confluent growth (4+); Semi-confluent growth (3+); 10 or more isolated colonies (2+); Less than 10 isolated colonies (1+); No growth (0).

TABLE XV

TESTS FOR INCREASED HEAT RESISTANCE ON EXPERIMENT HRT-2 "FIRST AND SECOND CYCLE" ISOLATES[a]

Isolate No.	Colony Development Patterns[b] on	
	BGA 35°C, 24 hrs	LA 35°C, 24 hrs
PBM 254,-271,-276,-284,-293, -298,-300	0	0
PBM 252,-279,-294	0	1+
PBM 250,-259,-267	0	2+
PBM 257,-277,-280,-281,-287, -289,-290,-295,-296	1+	1+
PBM 251,-253,-256,-265,-266, -270,-275,-283,-291,-292	1+	2+
PBM 282,-297	2+	1+
PBM 269,-299	2+	2+
PBM 255,-258,-260,-261,-273, -274,-278,-285,-301	2+	3+
PBM 263,-264	2+	3-4+
PBM 286,-288	3+	3+
Heated Controls		
SS32 (for PBM 250 to 284)	1+	2+
SS32 (for PBM 285 to 301)	1+	1+
SS 775W	4+	4+

[a] Cell suspensions in L-broth (ca $1x10^8$ cells /ml) were prepared from 24 hr, 35°C, shaker cultures and heated at 60°C for 2.5 minutes in sealed, capillary pipettes.

[b] No growth (0); less than 10 isolated colonies (1+);10 or more isolated colonies (2+); semi-confluent growth (3+); confluent growth (4+).

TABLE XVI

EFFECT OF INCUBATION TIME AND TEMPERATURE ON COLONY DEVELOPMENT PATTERNS FOR HEATED CELL-BROTH SUSPENSIONS OF SALMONELLA SENFTENBERG SPOTTED ON BRILLIANT GREEN AGAR MEDIUM

Isolate No.	No. of survivors per ml of heated cell suspension (L-Agar:24 hrs,35°C)	Colony development patterns on BGA medium at								
		9 hours			16 hours			44 hours		
		25°C	35°C	45°C	25°C	35°C	45°C	25°C	35°C	45°C
PBM 274	$1.3x10^5$	0	0	0	0	0	0	0	2+	0
PBM 275	$3.5x10^5$	0	0	0	0	0	0	1+	2+	0
PBM 276	10	0	0	0	0	0	0	1+	0	0
PBM 277	$8.3x10^4$	0	0	0	0	0	0	1+	2+	0
B 178	$1.5x10^4$	0	0	0	0	0	0	1+	2+	0
B 183	$4.0x10^4$	0	0	0	0	0	0	1+	1+	0
B 184	$1.5x10^5$	0	0	0	0	0	0	1+	2+	0
SS 32	$1.7x10^5$	0	0	0	0	0	0	1+	1+	0
SS 32[b]	$1.1x10^8$	4+	4+	4+	4+	4+	4+	4+	4+	4+
SS 775W	$9.0x10^7$	3+	4+	4+	4+	4+	4+	4+	4+	4+

[a] Cells from 35°C, broth shaker cultures were suspended in L-Broth at a final concentration of approximately $1x10^9$ per ml and heated at 60°C for 2.5 minutes in sealed, one ml pipettes. The colony development patterns and survivor concentrations appearing in the table are for the 10^{-1} dilutions of the heated cell suspensions.

[b] Unheated cell suspension

heating. The results of these tests appear in Table XXI, together with the results obtained with isolates from the fourth HRT experiment.

To simplify determinations and comparisons of increased heat resistance in the isolates, percent survivor(%S) and RIPS values were calculated from the survivor counts appearing in Tables XI, XII and XIII. These counts represent recovery of the organisms in L-Agar at 35°C following incubation for 48 hours (series No. 1), 72 hours (series No. 2), and 24 hours and 5 days (series No. 3). The respective survivor counts and %S values (100% = $1x10^9$ cells per ml) for the SS32 reference strain in test series 1, 2 and 3 were $1.1x10^5$ (0.011 %S), $7.6x10^5$ (0.076 %S), and $1.0x10^5$ (0.01 %S) after 24 hours and $9.6x10^5$ (0.096 %S) after 5 days. In relation to the %S value determined for the SS32 reference strain in each of the test series, RIPS values for the PBM isolates recovered in the "third cycle" of the second HRT experiment were as follows:

PBM 302 to 311 and SS775W (Table XI): 11.7, 31.7, 18.1, -0.254, 2.72, 22.6, 3.08, 3.63, 5.08, 1.36, and 9,100 (SS775W);

PBM 312 to 321 and SS775W (Table XII): 4.40, 3.21, 2.82, 2.16, 4.79, 3.34, 5.97, 5.58, 3.08, 2.95, and 1,210 (SS775W);

PBM 303 and 308 and SS775W after 24 hours and 5 days (Table XIII): 14.0 and 8.27; 3.7 and 3.48; and 4,800 and 510 (SS775W), respectively.

The RIPS values calculated for the isolates B190 to 194 after 24 hours and 5 days (Table XIII) were 6.3 and 2.23; 11.0 and 2.75; 0 and 0.021; 4.6 and 1.5; and -0.06 and -0.312 respectively. By definition, all RIPS values for the reference SS32 strain were "zero."

In comparing the RIPS values obtained for the isolates in the "third cycle" of the second HRT experiment it can be readily seen that these values and the range of these values varied for each of the test series. Since the organisms tested in each series were grown and processed under the same conditions, the major cause of these variations was attributed to the time period employed for recovery of the survivors in the "60°C - 2.5-minute" screening test. Variation in individual RIPS values are most noticeable for the positive control strain, SS775W, which was tested in each series. The values obtained for this organism were 9,100 (series No. 1); 1,210 (series No. 2); 4,800 at 24 hours and 510 at 5 days (series No. 3). The isolates PBM308 and PBM303 were tested in both series No. 1 and No. 3 and had RIPS values of 3.08 and 31.7 (series No. 1) and 3.7; 3.48 and 14.0; 8.27 (series No. 3), respectively. The ranges of the RIPS values for the PBM isolates in the first two series varied from approximately 1 to 32 (series No. 1) and 2 to 6 (series No. 2).

The %S value is based on a survivor count and can be quantitatively altered by any factor (susceptibility of the host, changes in processing time and temperature, initial cell concentration, recovery time and temperature, medium, etc.)

that will increase or decrease the numbers of survivors. The RIPS value, on the other hand, is calculated from the %S values determined for the isolate and the reference strain and can ostensibly remain uninfluenced by these factors if the survivor counts on which the %S values are based are altered proportionately. A case in point is a comparison of the RIPS values calculated for the isolates PBM 308 and 303 in the third test series which were based on survivor counts obtained for these isolates and for SS32 after 24 hours and 5-day incubation of the heated cell suspensions in L-Agar at 35°C. The survivor counts and %S values calculated for these times were 4.7×10^5 (0.047%S) and 4.3×10^6 (0.43%S) for PBM308; 1.5×10^6 (0.15%S) and 8.9×10^6 (0.89%S) for PBM303; and 1.0×10^5 (0.01%S) and 9.6×10^5 (0.096%S) for the reference strain. The RIPS values calculated for PBM308 for these two time periods were 3.7 and 3.48 and for PBM303 they were 14.0 and 8.27. The RIPS values for PBM308 remained essentially unchanged because the %S values for the isolate and the reference strain both increased by a factor of approximately 10 during the additional incubation period; the values for PBM303 differed because the %S value for this isolate only increased by a factor of 6.

A comparison of the RIPS value calculated for the isolates in the third test series (see above) indicate that the cells of the reference strain are more adversely affected by their initial exposure to the screening test temperature of 60°C for 2.5 minutes and require a longer incubation period to become visibly detected as survivors. Additional studies have shown

that the period of maximum recovery for heated SS32 cells in L-Agar at 35°C is at least 48 hours (Column VI, Table XVIII) and possibly 72 hours (Column III, Table XVIII).

In this experiment, RIPS values greater than 0.312 and calculated from survivor counts obtained at 48 hours or greater than 48 hours incubation at 35°C in L-Agar were felt to be significant and to indicate the approximate degree of increase in heat resistance of the isolate over that of the reference strain. The value 0.312 is the negative RIPS value determined for the isolate B194 and it is assumed in using this value that this isolate is equal in thermal resistance to SS32 and that this figure represents the margin of error that can be expected. A RIPS value of 0.8 was taken as the limiting value in the first HRT experiment (see Column III, Table X).

All of the "third cycle" isolates recovered in the second HRT experiment were found to be lysogenic (ly^+) and all the B isolates examined were determined to be non-lysogenic (ly^-) and susceptible to PSS775W (Table XXI). These isolates can be grouped according to their RIPS values as follows:

<0.49:	PBM305, B192, B194;
0.5 - 1.9:	PBM311, B193;
2.0 - 2.9:	PBM306, -314, -315, B190, B191;
3.0 - 3.9:	PBM308 (series Nos. 1 and 3), -309, -313, -317, -320, -321;
4.0 - 4.9:	PBM310, -312, -316, -319;
6.0 - 7.9:	PBM318;
8.0 - 9.9:	PBM303 (series No. 3);

TABLE XVII

EFFECT OF TIME AND TEMPERATURE ON RECOVERY OF S. SENFTENBERG STRAINS 32 AND 775W FROM HEATED CELL-BROTH SUSPENSIONS[a]

Incubation	Colony-forming units per ml of heated cell suspension					
	SS32			SS775W		
Temp / Time	25°C	35°C	45°C	25°C	35°C	45°C
12 hours	10	10	10	1.0×10^3	7.6×10^8	4.0×10^8
24 hours	10	160	10	7.6×10^8	7.6×10^8	4.5×10^8
48 hours	120	5.6×10^3	10	7.6×10^8	7.7×10^8	4.8×10^8
72 hours	1.2×10^4	1.1×10^4	10	7.8×10^8	8.1×10^8	5.1×10^8

[a] Cells from 24 hr, 35°C, broth shaker cultures were suspended in L-Broth at a final concentration of approximately 1×10^9 per ml and heated at 60°C for 2.5 minutes in sealed, capillary pipettes. Colony-forming units were determined in L-Agar.

TABLE XVIII

EFFECT OF INCUBATION TIME AND TEMPERATURE ON RECOVERY OF S. SENFTENBERG STRAINS 32 AND 775W FROM HEATED AND UNHEATED CELL-BROTH SUSPENSIONS

Incubation	Colony-forming units per ml of cell suspension											
	SS32						SS775W					
	Before Heating			After Heating			Before Heating			After Heating		
Time \ Temp	25°C	35°C	45°C	25°C	35°C	45°C	25°C	35°C	45°C	25°C	35°C	45°C
12 hours	5.4×10^{8}	9.2×10^{8}	$>1.0\times10^{8}$	2.0×10^{3}	3.7×10^{3}	1.6×10^{3}	5.1×10^{8}	8.9×10^{8}	7.8×10^{8}	9.5×10^{6}	1.5×10^{8}	1.5×10^{7}
				100	2.5×10^{3}	80						
	9.3×10^{8}	1.2×10^{9}	1.2×10^{9}	<10	<10	<10				1.0×10^{6}	1.4×10^{8}	1.8×10^{7}
24 hours	8.4×10^{8}	1.1×10^{9}	3.8×10^{8}	6.0×10^{3}	7.8×10^{3}	3.6×10^{3}	7.9×10^{8}	1.1×10^{9}	7.8×10^{8}	1.8×10^{8}	2.2×10^{8}	6.3×10^{7}
				5.0×10^{3}	5.8×10^{3}	340						
	1.1×10^{9}	1.2×10^{9}	1.3×10^{9}	10	<10	<10				2.4×10^{8}	2.1×10^{8}	8.7×10^{7}
48 hours	8.4×10^{8}	1.1×10^{9}	4.2×10^{8}	7.5×10^{3}	8.2×10^{3}	3.6×10^{3}	9.9×10^{8}	1.1×10^{9}	7.8×10^{8}	3.0×10^{8}	2.3×10^{8}	6.3×10^{7}
				8.3×10^{3}	5.8×10^{3}	340						
	1.1×10^{9}	1.2×10^{9}	1.3×10^{9}	20	<10	<10				3.4×10^{8}	2.1×10^{8}	8.7×10^{7}
72 hours	8.4×10^{8}	1.1×10^{9}	4.2×10^{8}	7.5×10^{3}	8.2×10^{3}	3.6×10^{3}	9.9×10^{8}	1.1×10^{9}	7.8×10^{8}	3.4×10^{8}	2.3×10^{8}	6.4×10^{7}
				8.3×10^{3}	5.8×10^{3}	340						
	1.1×10^{9}	1.2×10^{9}	1.3×10^{9}	20	<10	<10				3.4×10^{8}	2.1×10^{8}	8.7×10^{7}

[a] Cells from 24 hr, 35°C, broth shaker cultures were suspended in L-Broth at a final concentration of approximately 1×10^{9} per ml and heated at 60°C for 2.5 minutes in sealed, one ml pipettes. Colony-forming units were determined in L-Agar.

10.0 - 19.9: PBM302, -304; and

20.0 - 39.9: PBM303 (series No. 1), -307.

Some of the PBM isolates recovered in the first two cycles of the second HRT experiment were also shown to have increased in heat resistance (Table XV). The results of the screening tests for this characteristic indicated that eight of the "first cycle" isolates (30%) and seven of the "second cycle" isolates (25%) were significantly more heat-resistant than the SS32 reference strain (≥2+ reactions on both BGA and LA media in 24 hours at 35°C) but that they were not as heat-resistant as SS775W. Although tests for lysogenicity were not performed on any of the isolates recovered from these two cycles, the cells employed in the heating phases of these cycles were tested for lysogens and shown to be positive.

HRT Experiments Nos. 3 and 4

In the third and fourth transduction experiments an attempt was made to select "hr" transductions from heated "PBM" suspensions by recovering the heat-treated cells at an elevated temperature. After exposure to SS775W phage, SS32 cells were grown at 35°C for 3.32 generations, heat inactivated at 60°C for 3.0 minutes by the "tube" method, and plated on recovery medium (BGA or L-Agar). The plates were examined for survivors after incubation at 35°C and/or 45°C for various periods of time.

No isolates were recovered in the third HRT experiment because colonies failed to develop on the recovery medium during the incubation period. In this experiment, five ml of "PBM" and "B" broth cultures (5×10^9 heat-treated cells) were

plated separately onto BGA medium and incubated for five days at 45°C and then for five days at room temperature.

In the fourth HRT experiment, 2 ml quantities of the "PBM" and "B" broth cultures ($2x10^9$ heat-treated cells) were transferred each to two sets of L-Agar plates and two bottles of L-Broth for 48-hour incubation at 35°C and 45°C, respectively. The broth culture used in this experiment was a pool of three heated cultures and contained a total of $15x10^9$ heat-treated cells.

The results obtained in the fourt HRT experiment appear in Table XIX. In this experiment, 19 "PBM" isolates (PBM400-PBM418) and three control "B" isolates (B400-B402) were recovered; but only from the L-Agar plates incubated at 35°C for 48 hours. No colonies were seen on the plates incubated at 45°C. In like manner, growth occurred in the bottles of L-Broth inoculated with the heated "PBM" and "B" cultures and incubated for 48 hours at 35°C, but not at 45°C. Evidence for growth appeared much earlier in the bottle inoculated with the "PBM" culture (12 hours) than it did with the "B" culture (32 hours). The organisms recovered were assigned to Group H_1 and H_2, respectively, and given a "sample" number of I-60/35-3 indicating that they were obtained in the first cycle of growth at 35°C and thermal inactivation at 60°C, for 3.0 minutes (Tables VIII, IX).

Tests for thermal resistance and lysogeny were performed on all of the isolates recovered in the fourth experiment.

TABLE XIX

RECOVERY OF SURVIVORS FROM EXPERIMENT HRT-4
ON L-AGAR AND IN L-BROTH AT 35 AND 45 C

Survivors	Recovery Temperature	Recovery Media for Heated Cell Suspensions[a]											
		L-Agar (48 hrs.)											L-Broth (48 hrs.)
		Colony-forming units per volume of suspension plated											Growth (+) of 2.0 ml of heated suspension in 50 ml of medium
		0.2 ml										2.0 ml	
		1	2	3	4	5	6	7	8	9	10	Total	
PBM	35°C	7	4	3	0	1	1	1	1	1	0	19	+ 12 hrs.
	45°C	0	0	0	0	0	0	0	0	0	0	0	0
B	35°C	0	0	1	0	0	0	1	1	0	0	3	+ 32 hrs.
	45°C	0	0	0	0	0	0	0	0	0	0	0	0

[a] Suspensions of SS32 cells (B) and mixed suspensions of PSS775W and SS32 cells (PBM) were heated at 60°C for a period of 3 minutes. The bacterial cells were in the late log phase of growth at a concentration of approximately 0.8×10^9 per ml prior to heating.

The results of these tests appear in Tables XX and XXI, respectively, and the isolates are identified in Table IX. Isolates were screened for thermal resistance by the "spot" plate method and assayed for lysogeny by the qualitative test for phage described in the section of "Materials and Methods." Table XX shows the colony development patterns obtained for the isolates and heated controls of SS32 and SS775W after 12 and 24 hours incubation on BGA medium at both 35°C and 45°C. All test suspensions of the organisms were prepared from 24-hour-35°C broth shaker cultures and contained $1x10^9$ viable cells per ml when heated at 60°C for 2.5 minutes by the "sero-pipette" method. For comparison with the heated controls of SS32 and SS775W, the isolates have been grouped and arrayed according to the similarity of their colony development patterns.

Tests for lysogenicity (Table XXI) indicated that 14 of the 19 "PBM" isolates recovered in the fourth experiment (PBM405 - PBM418) contained SS775W prophage and that only one of the 3 "B" isolates was lysed by PSS775W. The remaining 5 "PBM" isolates (PBM400 - PBM404) and 2 "B" isolates (B401 and B402) yielded negative tests for both phage infectivity and immunity. These isolates were later shown by microscopic examination and biochemical and serological tests to be contaminants.

The results of the screening tests for heat resistance indicated that 9 of the 14 "PBM" isolates (64%) were more heat-resistant than the SS32 control ($\geq$2+ reactions on BGA medium

TABLE XX

TESTS FOR INCREASED HEAT RESISTANCE ON EXPERIMENT HRT-4 ISOLATES[a]

Isolate No.	Colony development patterns on BGA medium at			
	12 hours		24 hours	
	35°C	45°C	35°C	45°C
PBM 400,-401,-402 -403,-404 B 400,-401,-402	0	0	0	0
PBM 410,-415	0	0	1+	0
PBM 411	0	0	2+	0
PBM 413,-416	1+	0	2+	0
PBM 405,-406,-407, -409,-412,-414	2+	0	2+	0
PBM 408,-418	2+	0	2+	0
PBM 417	3+	0	3+	0
Heated Controls				
SS32(PBM 400 to 416)	0	0	0	0
SS32(others tested)	0	0	2+	0
SS775W	4+	4+	4+	4+

[a] Cells from 24 hr, 35°C, broth shaker cultures were suspended in L-Broth at a final concentration of approximately $1x10^9$ per ml and heated at 60°C for 2.5 minutes in sealed, one ml pipettes.

TABLE XXI

TESTS FOR LYSOGENY ON EXPERIMENT HRT-2 AND HRT-4 ISOLATES

Isolate No.	Spot/Lawn Tests for Phage Infectivity and Immunity	
	Isolate/SS32	PSS775W/Isolate
PBM 302 - PBM 321 PBM 405 - PBM 418 SS775W (Control)	+	-
B 190 - B 194 B 400 SS32 (Control)	-	+
PBM 400 - PBM 404 B 401 - B 402	-	-

+ = lysis of lawn culture

- = no lysis

in 12 hours at 35°C) but less heat-resistant than SS775W. Not only did the heated test suspensions of the isolates fail to achieve the colony development patterns of the heated SS775W control at 35°C but their recovery was inhibited at 45°C (Table XX).

The Effect of Incubation Time and Temperature on the Recovery of Salmonella senftenberg Strains 32 and 775W from Heated and Unheated Cell-Broth Suspensions

In the screening tests employed in this study to determine heat resistance, both the "plate count" method and the "spot" plate method were used to recover and evaluate survivors from heated cell-broth suspensions. The "plate count" method was used to test "first and second" cycle isolates obtained in the first HRT experiment (HRT-1) and also "third cycle" isolates obtained in the second HRT experiment (HRT-2). In these tests all survivors were recovered in or on L-Agar following incubation at 35°C but the time allowed for recovery was not always the same. Recovery times varied from 72 hours (Tables X, XII) to 48 hours (Table XI) to 24 hours and 5 days (Table XIII). After preliminary experiments had been carried out with heated and unheated suspensions of SS32 and SS775W (Table XIV), the "spot" plate method was introduced into this study as a simple and rapid method to recover and evaluate survivors in the screening test for heat resistance. This method was first used in testing "first and second cycle" isolates obtained in the second HRT experiment (Table XV). In these tests both BGA medium and L-Agar were used as the

recovery medium and 24 hours and 35°C as the recovery time and temperature, respectively.

The "spot" plate method was used in the initial experiment designed to determine the effect of time and temperature on recovery of strains of Salmonella senftenberg from heated cell-broth suspensions. The results of this experiment appear in Table XVI where colony development patterns for "second cycle" HRT-2 isolates (PBM274 - PBM277) and the controls (B178, B183 - B184) are compared with patterns obtained for heated suspensions of SS32 and SS775W and for an unheated suspension of SS32 at 25°C, 35°C, and 45°C following incubation for 9, 16, and 44 hours. The colony development patterns are also compared with survivor counts of the heated suspensions in L-Agar at 35°C for 24 hours. In this experiment the test suspensions were prepared from 24-hour-35°C broth shaker cultures and contained $1x10^{9}$ viable cells per ml when heated at 60°C for 2.5 minutes by the "sero-pipette" method. It should be noted that the colony development patterns and survivor counts appearing in this table are for the 10^{-1} dilutions of the heated cell suspensions.

The outstanding finding in this experiment was the fact that the heat-resistant donor strain, after being heated in broth for 2.5 minutes at 60°C, formed a confluent colony on BGA medium at all temperatures, including 45°C after 9 hours incubation, while the "PBM" and control "B" isolates and the heated SS32 strain failed to develop even a single colony on

this medium after 16 hours incubation at any one of the three temperatures. This observation was inconsistent with the fact that survivor counts, as determined in L-Agar after 24 hours incubation at 35°C, ranged between 1.5×10^4 to 3.5×10^5 per ml of the 10^{-1} dilution plated and should have (considering a loop dilution factor of 230) resulted in between 65 to 1500 viable cells being inoculated onto the surface of the medium. One explanation for failure of these cells to develop into isolated or confluent colonies was, of course, the possibility that the BGA medium was inhibiting their growth.

The "spot" plate method is, at best, a semi-quantitative method of determining survivors and in continuing this study it became necessary to change to the quantitative "plate count" method in order to obtain more substantial data. In an effort to concentrate solely on the effect of time and temperature on recovery of heat-resistant and heat-sensitive survivors, a decision was made to limit the test organisms to SS32 and SS775W and to use L-Agar as the recovery medium.

Two experiments were performed to study the effect of the parameters of incubation time and temperature on recovery of SS32 and SS775W from heated cell-broth suspensions (Tables XVII, XVIII). In these experiments the test suspensions were prepared from 24-hour-35°C broth shaker cultures and contained approximately 1×10^9 viable cells per ml when heated at 60°C for 2.5 minutes by the "sero-pipette" method. After heating, the suspensions were diluted 10^{-1} in 3.6 ml of

L-Broth to prepare the first decimal dilution and from this dilution serial, decimal dilutions were made in 4.5 ml of broth to a final dilution of 10^{-7}. Each dilution of the heated suspension was plated in triplicate in L-Agar for incubation at 25°C, 35°C, or 45°C. Plates were counted after incubation at these temperatures for 12, 24, 48, and 72 hours and survivors were recorded as colony-forming units per ml of cell suspension.

Table XVII shows the effect of incubation time and temperature on recovery of SS32 and SS775W from heated cell-broth suspensions only. In this experiment, a single test suspension of each organism was heated at 60°C for 2.5 minutes and processed for survivor counts at 25°C, 35°C, and 45°C. Table XVIII, on the other hand, shows the effect of the two parameters on recovery of these organisms from both heated and unheated cell-broth suspensions. For SS32, three heated and two unheated test suspensions were processed for survivor counts at the three experimental temperatures; for SS775W, only two heated suspensions and one unheated suspension were processed.

In interpreting the results obtained in these two experiments, the maximum number of survivors at all three incubation temperatures employed (25°C, 35°C, and 45°C) was taken as the number of cell units forming detectable colonies in the plating medium in 72 hours. Using 35°C as a reference temperature, the maximum number of survivors recovered in this time period

was found to be much less for the heated SS32 cells (0.001 %S, Table XVII; 0.0006 %S, Table XVIII) than for the heated SS775W cells (81.0 %S, Table XVII; 25.0 %S, Table XVIII). In comparison with this reference temperature, maximum recovery of the heated cells of both strains was approximately the same at 25°C but lower at 45°C (ca. 70% lower for the SS775W cells and between 95.0 to 99.9% lower for the SS32 cells). For the unheated cells of both strains, the total numbers of survivors were the same with the possible exception of the number of SS775W cells recovered at 45°C.

Maximum recovery of the heated and unheated cells of both strains was observed in 24 hours at 35°C and 45°C and in 48 hours at all three incubation temperatures (Table XVIII). It also occurred after longer periods of incubation. The results in Table XVII indicate that maximum recovery of the heated SS32 cells took place in 48 to 72 hours at 25°C and 35°C and that recovery of these cells was almost completely inhibited at 45°C; this is in contrast to the rapid recovery with only slight inhibition seen for the heated SS775W cells. While this tendency for heated SS32 cells to lose their ability to grow at 45°C is only suggested in the results of the follow-up experiment recorded in Table XVIII, it has been confirmed in other experiments (see Tables XVI, XIX, XX, and XXIII). When the times for complete or partial recovery of the maximum number of heated SS32 and SS775W cells at the various incubation temperatures are compared, it can be seen that the heated and unheated cells of both strains recovered faster at 35°C

than at 25°C and that the heated SS32 cells recovered much more slowly at 25°C and 35°C than the heated cells of SS775W.

The results of these studies on the effect of incubation time and temperature on the recovery of heated cells of the heat-sensitive SS32 strain and the heat-resistant SS775W strain indicate that the effect of heating at inactivation temperatures on the heat-sensitive strain, in addition to completely destroying its ability to recover under normal conditions for growth, is to delay its recovery at optimum and suboptimum growth temperatures and to inhibit its ability to grow at an elevated temperature. Organisms that require an increase in time to grow and resume cell division following exposure to sublethal heat treatment were considered by Eijkman (1908) as "injured" cells.

To pave the way for future discussion on the nature and origin of heat resistance in SS775W, it seems justified at this time to reexamine the results of the effect of time and temperature on the recovery of heated SS32 and SS775W cells appearing in Table XVII in terms of cell injury and death. The following interpretation can be made from a comparison of the survivor counts obtained for heated SS32 cells at all three recovery temperatures (25°C, 35°C, and 45°C) in 12, 24, and 72 hours: cells that recovered at all three temperatures in 12 hours either failed to receive or resisted heat treatment or were quickly able to repair any thermal damage that was done; those that recovered in 72 hours at 25°C and 35°C

but not at 45°C were "injured" cells; those that recovered in 24 hours at 35°C were slightly "injured" cells; and cells that did not recover in 72 hours were fatalities in the sense that damage at some functional site in the cell was extensive and beyond repair. Using the same recovery times and temperatures for comparison of the survivor counts obtained with heated SS775W cells it can be seen that ca 21 percent of the population of SS775W were fatalities and that ca 30 percent of the survivors were injured cells that developed slowly at 25°C in 12 hours and also failed to develop at 45°C in 72 hours. Maximum recovery of the SS775W cells at 25°C and 35°C and almost maximum recovery at 45°C in 24 hours indicates that most of the surviving cell population were either unaffected (completely resistant) or only very slightly injured by the heat treatment (60°C, 2.5 minutes) they received.

With the exception of one test result, the results of these two experiments confirm the previous finding that growth of heat-sensitive cells, following heat-treatment or heat-shock, is inhibited at an elevated temperature of 45°C. This finding led to the use of 45°C as a selective elevated temperature for testing the thermal resistance of salmonellae and recovering them from mixed cultures and cell-suspensions. Temperatures of 45°C were used in an attempt to select "hr" transductants from heated "PBM" suspensions in HRT experiments Nos. 3 and 4 (see Table XIX), for testing HRT-4 isolates recovered from heated suspensions at 35°C but not at 45°C (Table

XX), and for comparing the heat resistance of SS32, SS775W, SSD8, and SS331W (Table XXIII).

Isolation and Characterization of a Heat-Resistant Mutant of Salmonella senftenberg Strain 32

Isolation

Identification of Experiment HRT-2 "Samples" and "Sample" Cultures. During the phases of thermal inactivation in the second HRT (HRT-2) experiment samples were removed at various times from the cell-broth suspensions being heated at 55°C. For each sample taken--a portion was removed for plating to determine the number of survivors, a portion was inoculated into broth and immediately incubated at 35°C, and the remainder of the sample was stored at 4°C.

Although the designations "stored" sample and "incubated" sample are perhaps more accurate descriptive terms, reference has been made in this presentation to the portion stored at 4°C as the "sample" and to the portion inoculated into broth and incubated at 35°C as the "sample" culture. An attempt was made to use ".01 sample" cultures throughout this particular transduction experiment and these have been defined as overnight cultures of samples that contained approximately 0.01 percent survivors. In like manner, reference may also be made to portions of the unheated samples stored at 4°C as the "100 sample" and the portion inoculated in broth and incubated at 35°C as the "100 sample" culture. Alternately, the unheated sample could be referred to as the "zero sample" (i.e., the

sample representing "zero" time) and the unheated, incubated sample as the "zero sample" culture.

The unheated sample ("100 sample" or "zero sample") is a 10^{-2} dilution of the same broth culture or diluted broth culture from which one ml was removed and inoculated into 99 ml of broth preheated to 55°C. It is from this sample that colony counts were made to determine 100 percent survivors at "zero" time for the construction of thermal inactivation curves. In the heating phases of the first two cycles the broth cultures used were in the late-log phase of growth and in the third cycle they were in the stationary phase and had to be diluted 10^{-1} for inoculation into the heated medium. The unheated sample in the third cycle was prepared from a 10^{-2} dilution of the primary 10^{-1} dilution.

The results of the screening tests for thermal resistance on the first, second, and third cycle isolates recovered from the HRT-2 experiment are shown in Tables XI, XII, XIII, XV, and XVI. Similar tests were also carried out on the unheated, test (PBM) and control (B) samples and "sample" cultures obtained in the first "growth and heating" cycle of this experiment, and these results appear in Table XXII. They are presented separately because isolates were not recovered from these samples during the phases of thermal inactivation.

Tests for Thermal Resistance. The samples and "sample" cultures containing the unheated test and control cells were stored for seven weeks at 4°C before cell-broth suspensions

TABLE XXII

TESTS FOR INCREASED HEAT RESISTANCE ON UNHEATED SAMPLES AND SAMPLE CULTURES OBTAINED IN THE FIRST "GROWTH AND THERMAL INACTIVATION" CYCLE OF HRT EXPERIMENT NO. 2

Sample no.	No. of survivors per ml of heated cell-broth suspension[a] in L-Agar at 35 C after			
	24hrs	48hrs	72hrs	5days
First Heating[b]				
PBM: I-55/35-0(culture)	1.8×10^6	-	-	6.9×10^6
PBM: I-55/35-0(sample)	6.2×10^6	-	-	9.6×10^6
B: I-55/35-0(culture)	8.8×10^5	-	-	1.7×10^6
B: I-55/35-0(sample)	2.3×10^6	-	-	4.6×10^6
SS32	1.3×10^6	-	-	3.3×10^6
SS775W	6.7×10^8	-	-	8.4×10^8
Second Heating[c]				
PBM: I-55/35-0(culture)	5.6×10^5	8.3×10^5	8.5×10^5	8.5×10^5
PBM: I-55/35-0(sample)	1.4×10^4	2.7×10^4	2.8×10^4	3.2×10^4
B: I-55/35-0(culture)	$>1.0 \times 10^8$[d]	$>1.0 \times 10^8$	$>1.0 \times 10^8$	$>1.0 \times 10^8$
B: I-55/35-0(sample)	1.0×10^6	1.7×10^6	1.7×10^6	1.7×10^6
SS32	2.2×10^4	4.6×10^4	9.0×10^4	9.6×10^4
SS775W	8.1×10^8	9.0×10^8	9.0×10^8	9.0×10^8

[a] Test suspensions were prepared from 10^{-1} dilutions of 24hr-35 C, broth shaker cultures and contained ca, 1×10^9 viable cells per ml when heated at 60 C for 2.5 min by the "sero-pipette" method.

[b] Sample and "sample" cultures were stored for 7 weeks at 4 C before test suspensions were prepared for the first heating.

[c] Cells that survived the first heating were regrown in L-Broth for 48 hrs and stored for 3 days at 4 C before test suspensions were prepared for the second heating.

[d] Estimated value.

were prepared for the screening test for heat resistance. These suspensions were 10^{-1} dilutions of 24-hour-35°C, broth shaker cultures and contained ca, $1x10^{9}$ viable cells per ml when heated at 60°C for 2.5 minutes by the "sero-pipette" method. After heating, a 0.4 ml portion of the test suspension was removed from each pipette to prepare dilutions for plating in L-Agar and the remainder of the suspension was inoculated into 5 ml of L-Broth and reincubated at 35°C on a shaker. The plates prepared from the dilutions were incubated at 35°C for a total of five days and counted at this time and also after 24 hours. The survivor counts obtained for the samples and "sample" cultures in the first screening test for thermal resistance are shown in Table XXII.

The broth tubes containing the heated suspensions were incubated at 35°C for 48 hours. All tubes were positive for growth at the end of this period; only the tube containing the B: I-55/35-0 (culture) was positive at 24 hours. These cultures were stored at 4°C for three days before transfers were made to broth to prepare the 24-hour-35°C, cultures required in the screening test. On the fourth day, suspensions of the samples and "sample" cultures were made and heated as before. The heated suspensions were processed for incubation in L-Agar at 35°C for five days with survivor counts recorded at that time and after 24, 48, and 72 hours. The counts obtained for the samples and "sample" cultures in the second screening test for heat resistance are also given in Table XXII.

The concentration of cells in all of the unheated test suspensions were estimated from percent transmittance readings observed at 425 mμ ($\%T_{425}$) for the 10^{-1} dilutions of the test suspensions in broth but are not included in the table. The $\%T_{425}$ readings for all of the dilute suspensions ranged from 79 to 81 ($80\%T_{425} = 1x10^{8}$ CFU per ml).

The most significant finding from the screening tests for thermal resistance carried out on the unheated, test (PBM) and control (B) samples and "sample" cultures obtained in the second HRT experiment (Table XXII) was that the B: I-55/35-0 "sample" culture apparently contained cells that were comparable in heat resistance to SS775W.

Preliminary Tests and Presumptive Identification. To determine if the cells in the B: I-55/35-0 culture were those of SS32, SS775W, or an unwanted contaminant, the culture was streaked immediately to BGA and L-Agar media to obtain isolated colonies.

Isolates were obtained on both plates following incubation and it was noted at the time that those on the BGA plate had the characteristic reaction and appearance of colonies of SS32 and SS775W on this medium. A portion of one of the colonies on the BGA plate was transferred to an agar slant and maintained as a stock culture.

In preliminary characterization tests, the organism was found to agglutinate in Salmonella "O" Group E_4 antiserum and to resemble SS32 rather than SS775W in its reaction on Kliger's

Iron Agar at 35°C--it produced an alkaline reaction on the slant portion of this medium and H_2S and acid but no gas in the butt in 24 hours; SS775W produced an alkaline slant and acid with gas in the butt portion in 24 hours but failed to produce H_2S in 48 hours.

An additional screening test for thermal resistance was conducted with test suspensions of the isolate, SS32, and SS775W using the "spot" plate method to recover survivors. Both the isolate and SS775W gave a 4+ colony development pattern on BGA media after 24 hours at 35°C and 45°C and SS32 gave a 4+ reaction pattern at 35°C but only a 1+ pattern at 45°C. The test suspensions contained $1x10^9$ cells per ml before heating.

These preliminary tests offered presumptive evidence that the isolate, referred to as the SS331W strain of Salmonella senftenberg, was a mutant strain of SS32 that had acquired the property of heat resistance.

Characterization

To confirm the hypothesis that the isolate identified as SS331W was a heat-resistant mutant of SS32, tests were performed for thermal resistance, lysogenicity, and certain biochemical characteristics on the "wild type" SS32 strain, the suspected mutant SS331W strain, and the known heat-resistant SS775W and SSD8 strains.

Thermal Resistance. Table XXIII shows a comparison of the colony development patterns of dilutions of heated test suspensions of SS775W, SSD8, SS331W, SS32, and an unheated suspension

TABLE XXIII

COMPARISON OF HEAT RESISTANCE IN SELECTED *SALMONELLA SENFTENBERG* STRAINS[a]

Cell Suspension Dilutions	Colony Development Patterns on BGA Medium (24 hrs, 35°C and 45°C)									
	Heated Suspensions								Unheated	
	SS775W		SSD8		SS331W		SS32		SS32	
	35	45	35	45	35	45	35	45	35	45
10^0 ($1x10^9$ cell/ml)	4+	4+	4+	4+	4+	4+	4+	0-1+	4+	4+
10^{-1}	4+	4+	4+	4+	4+	4+	3+	0	4+	4+
10^{-2}	4+	4+	4+	4+	4+	4+	2+	0	4+	4+
10^{-3}	4+	3-4+	4+	4+	4+	4+	1+	0	4+	4+
10^{-4}	3+	2-3+	3+	2+	3+	2+	0	0	3-4+	3+
10^{-5}	2+	1-2+	2+	1+	1+	1+	0	0	2-3+	2+
10^{-6}	1+	0-1+	1+	0	0-1+	0	0	0	1+	1+
10^{-7}	0	0	0	0	0	0	0	0	0	0
Log of 2+ Endpoint Dilution	-5	-4.7	-5	-4	-4.7	-4	-2	>0	-5.7	-5

[a]Cells from 24 hr. (35°C) broth cultures were suspended in L-Broth (10^0 dilution = 1×10^9 cells/ml) and heated at 60°C for 2.5 minutes in 1 ml pipettes sealed at the tip. Dilutions of the heated suspensions were "spotted" on the recovery medium with a 3mm wire loop that transferred a volume of ca 1/230 ml.

[b]Numerical Key: Confluent growth (4+); Semi-confluent growth (3+); 10 or more isolated colonies (2+); Less than 10 isolated colonies (1+); No growth (0).

of SS32 on BGA media after 24 hours at 35°C and 45°C. The test suspensions were 10^{-1} dilutions of 24-hour-35°C, broth shaker cultures and contained ca, $1x10^{9}$ viable cells per ml when heated at 60°C for 2.5 minutes by the "sero-pipette" method. The dilutions of the suspensions were made in L-Broth and "spotted" onto the surface of the media with a wire loop.

By determining or estimating the dilutions of the heated suspensions that gave a 2+ colony development pattern, survivor values were obtained which indicated the heat resistance of the organisms. These values were expressed as the "log of the 2+ endpoint dilution" and were used to compare the heat resistance of the strains tested. For suspensions that gave 2-3+ pattern at one decimal dilution (10^{-n}) and a 1-2+ pattern at the next higher dilution ($10^{-(n+1)}$), the log of the 2+ endpoint was estimated as $10^{-(n+0.74)}$; for example, if a 2-3+ pattern was obtained at 10^{-4} dilution and a 1-2+ pattern was obtained at a 10^{-5} dilution, then the log of the 2+ endpoint dilution was estimated as being mid-way between -10,000 and -100,000 or -55,000. Since the log of 55,000 is -4.74 then this was the value recorded. Endpoints estimated in this manner appear in the table only to the tenth decimal place.

Lysogeny. The four Senftenberg strains were assayed for the character of lysogeny by the qualitative test for phage described in the "Materials and Methods" section. The results of these tests appear in Table XXIV.

Biochemical Tests. Tests to determine the biochemical characteristics of SS32, SS775W, and SSD8 were carried out

TABLE XXIV

TESTS FOR LYSOGEN PRODUCTION IN HEAT-RESISTANT STRAINS OF SALMONELLA SENFTENBERG

Spot \ Lawn	SS775W	SSD8	SS331W	SS32
SS775W	-	-	+	+
SSD8	-	-	+	+
SS331W	-	-	-	-
SS32	-	-	-	-

+ = lysis of lawn culture

- = no lysis

earlier in this study with the following reactions observed at 35°C.

Positive Reactions (<48 Hours). Tests for arginine dihydrolase, catalase, methyl red, NO_3 reduction, motility at 35°C and 25°C were positive; arabinose, dextrin, dextrose, dulcitol, citrate, galactose, levulose, maltose, mannitol, mannose, melibiose, rhamnose, sorbitol, trehalose, and xylose were utilized.

Delayed Positive Reactions. Esculin was utilized in 3 days and cellibiose in 5 days.

Negative Reactions. Tests for acetylmethylcarbinol, cytochrome oxidase, indole, phenylalanine deaminase, and urease were negative; alpha-methylglucoside, adonitol, amygdalin, erythritol, gluconate, glycogen, inositol, inulin, lactose, malonate, melizitose, raffinose, salicin, sorbose, and sucrose were not utilized in 14 days.

Differential Reactions. SS775W and SSD8 utilized dextrose, dulcitol, sorbitol, trehalose, and xylose with the production of acid and gas and were lysine decarboxylase positive and H_2S (KIA) negative. SS32 utilized the above substrates with the production of acid but no gas and was lysine decarboxylase negative and H_2S (KIA) positive.

After isolation of the SS331W strain the biochemical tests differentiating SS775W and SSD8 were repeated using all four strains. The results of these tests appear in Table XXV.

Results of the tests to characterize the isolate, SS331W, indicated that it was a heat-resistant mutant of SS32 whose level of thermal resistance was equal to that of the SS775W and SSD8 strains (Table XXIII). Like its "wild-type" parent, the SS331W strain was lysed by the bacteriophages of SS775W and SSD8 (Table XXIV) and could be biochemically identified with SS32 and differentiated from the other heat-resistant strains by its inability to decarboxylate lysine and its ability to produce H_2S in KIA medium and to utilize dextrose, xylose, trehalose, sorbitol and dulcitol with the production of acid but no gas (Table XXV).

Several tests for glycerol utilization by strains SS775W, SSD8, SS32, and SS331W were performed using Bacto-Purple Broth Base medium (Difco Product No. 0227) with one percent glycerol added instead of Stern's Glycerol Fuchsin medium (Stern, 1916), which is the standard medium employed for determining glycerol utilization (Kauffmann, 1961 and 1966; Edwards and Ewing, 1962). The results obtained with the substituted glycerol medium were variable and inconclusive and were not reported in this study. Ng et al. (1969), however, have reported that this substrate is not utilized by SS775W. Attention is called to this fact because it appears that the SS775W strain, and possibly the SSD8 strain, have biochemical

TABLE XXV

BIOCHEMICAL DIFFERENTIATION OF THE LYSOGENIC HEAT-RESISTANT STRAINS OF SALMONELLA SENFTENBERG AND THE WILD TYPE SS32 STRAIN AND ITS HEAT-RESISTANT MUTANT

Substrate or Test	SS775W (hr^+, ly^+)	SSD8 (hr^+, ly^+)	SS32 (hr^-, ly^-)	SS331W (hr^+, ly^-)
Dextrose	AG	AG	A	A
Xylose	AG	AG	A	A
Trehalose	AG	AG	A	A
Sorbitol	AG	AG	A	A
Dulcitol	AG	AG	A	A
Lysine Decarboxylase	+	+	-	-
H_2S (KIA)	-	-	+	+

Abbreviation Key:

hr^+, hr^- = heat resistant, non-heat resistant.

ly^+, ly^- = lysogenic, non-lysogenic.

AG, A = acid and gas or acid production within 24 hrs (35^oC).

+, - = Tests positive within 24 hrs (35^oC) or negative at five days.

KIA = Kliger's Iron Agar. The slant/butt reactions on this medium were Alk/AG for H_2S^- strains and Alk/A for H_2S^+ strains. Strains H_2S^- at 5 days were H_2S^+ at 7 days.

properties that identify them as Salmonella senftenberg var. newcastle (Kauffmann, 1931; Warren and Scott, 1930; Subcommittee on Salmonella, 1934) which is a biochemical variant of S. senftenberg that fails to produce H_2S and to utilize glycerol (Kauffmann, 1961). It should be pointed out that the SS32 and SS331W strains, for the differential reactions being reported, also appear to be biochemical variants of this species of Salmonella that fail to decarboxylate lysine and produce gas from glucose.

DISCUSSION

Isolation and Characterization of Salmonella senftenberg Lysogens

The main purpose of this phase of the study was to show that the reported heat-resistant strains of Salmonella senftenberg, SS775W and SSD8, carried temperate phages and that at least one of the indicator strains employed for their detection could also be used in experiments designed to determine if the character of heat resistance is a transferrable trait in salmonellae. To accomplish this objective a relatively large number of strains of S. senftenberg were examined for the presence of lysogens and for lysogen susceptibility.

Sixty-four American and 23 British strains of S. senftenberg (Tables I, II), including SS775W and SSD8 and the LT-2 strain of Salmonella typhimurium (STLT2), were tested for lytic activity on strains SS5, SS7, SS32, SS775W, SSD8 and STLT2 (Table III). In preliminary experiments, which established the feasibility of this study, it was shown that SS775W and SSD8 carried temperate phages for the SS5, SS7 and SS32 strains. Of the 87 strains tested, approximately 60 percent (58.6%) were shown to contain lysogens for the indicator strains by the scoring and test methods employed. Failure of an indicator strain to be lysed by a temperate phage spontaneously released from one of the test strains

was interpreted to mean that either the indicator strain did not have a receptor site for attachment of the phage ("receptor site" immunity) or that it already contained a resident prophage that was producing an immunity repressor substance which prevented multiplication of any related superinfecting phage ("prophage" immunity).

When supernatants of the indicator strains were spotted on lawn cultures of the same strains, three of the six strains tested were shown to be lysogenic. SS775W and SSD8 carried lysogens to which SS7 and SS32 were susceptible and SS5 carried a lysogen for SS7. The fact that these lysogens were temperate phages was demonstrated by their ability to form isolated and turbid plaques with the indicators in culture "cross-streak" tests.

Although the heat-resistant strains were lysogenic, they were themselves susceptible to phages carried by SS24, SS25, SS26 and SS27 indicating that these four strains carried phages unrelated to those of SS775W and SSD8. The "L.SS24 to L.SS27" group of phages are of particular interest because they lysed all of the Senftenberg indicator strains with the exception of SS7 which was susceptible to only one group member, L.SS25. Some of the Senftenberg lysogens were found to infect only one of the indicator strains while others lysed SS32 and one other indicator strain.

While it is easy to explain that the lysogens capable of infecting the lysogenic strains SS775W, SSD8 and SS5 were unrelated and therefore immune to the repressor substance produced

by the prophages and to explain that phages which would infect only one indicator strain and no other required specific sites for attachment possessed only by these strains, it could not be determined whether the immunity of SS775W, SSD8 and SS5 to many of the lysogens that infected SS32 was due to the presence of their prophage or to the absence of essential receptor sites.

SS32 was chosen as the recipient strain to demonstrate that heat resistance can be transferred by bacteriophage in the genus Salmonella. In preliminary screening tests for heat resistance this strain was shown to be relatively heat-sensitive (hr^-) compared to SS775W and this initial observation was repeatedly borne out in subsequent screening tests (Tables X to XX, XII, XIII). There is good evidence that this strain is also non-lysogenic (ly^-). Such evidence is based on the results of lysogen susceptibility and immunity tests (Table III) and on UV induction studies (Figure 5). SS32 was found susceptible to lysogens carried by 37 (42.5%) of the 87 strains tested and produced no plaques on any of the other indicator strains. Eighteen of these strains carried lysogens specific for SS32. In addition, when this strain was employed as a lawn culture in the qualitative assay test for phage, it failed to produce lysogens that lysed the surface colonies developing from bacteria present in the unfiltered supernatants of test strains that were spotted and scored negative for lysogens. In UV induction profile studies, this strain

failed to lyse after exposure to a dosage of ultraviolet irradiation sufficient to interrupt DNA synthesis and induce excision of the SS775W prophage (see Figure 5).

Spot tests for lysogen immunity and susceptibility revealed that the temperate phages carried by SS775W and SSD8 are related since each of these two strains was immune to lytic infection with the phage carried by the other. Common receptor sites for these two phages were found on the SS32 and SS7 strains but not on SS5. The ability of L.SS18 to lyse only SS775W and SS32 and for L.SS84 to lyse only SSD8 and SS32 is indicative of either a difference in the structure of the monomeric molecules or in the arrangement of similarly structured molecules at the same or at different receptor sites. There is evidence from work with the "T" phages of Escherichia coli that some of these phages (T_2, T_5, T_6) attach to the outer lipoprotein layer of the cell wall while the others (T_3, T_4, T_7) attach to the heptose portion of the inner lipopolysaccharide which is a common but not exclusive receptor site for this particular subgroup (see Hayes, 1964). Presumably, a similar situation could exist for the phages of S. senftenberg; their preferred site of attachment being one or a specific combination of the common basal sugars, the cell wall lipoprotein, or some other surface component of the host cell (envelope, fimbriae, or flagella) rather than to the sugars comprising its O-specific sidechains. Such a variety

of possibilities for attachment would also explain the susceptibility of S. typhimurium LT-2 to lysogens produced by SS75 and SS83.

In addition to its spectrums of lytic and immune response activity with the indicator strains and of susceptibility to infection with lysogens from the 87 strains of S. senftenberg that were tested, the temperature phage of SS775W has been shown to possess other characteristics. It was found, for example, that PSS775W is inducible with ultraviolet light, requires the presence of divalent cation for adsorption, and that its attachment to the host strain can be prevented with sodium citrate. It was also found that magnesium ion increases the efficiency of plaque formation and that the phage is moderately sensitive to chloroform and not readily adsorbed by cellulose triacetate membranes.

In summary, the experimental results obtained in this phase of the study showed that the heat-resistant SS775W and SSD8 strains of Salmonella senftenberg contain related temperate phages and that the SS32 and SS7 strains could be employed for their detection. SS32 was selected as the recipient strain to demonstrate that a genetic trait for heat resistance can be transferred by bacteriophage in the genus Salmonella and SS775W was chosen as the donor strain for the heat-resistant marker. The SS32 strain was shown to be heat-sensitive (hr^-), apparently non-lysogenic (ly^-), and susceptible to lysogenic infection with PSS775W. Tests for the susceptibility

and immunity of five selected Senftenberg strains (SS5, SS7, SS32, SS775W and SSD8) to lysogens carried by 87 Senftenberg strains, including those mentioned, indicate that a successful "lytic" or "lysogenic" typing scheme could easily be developed for strains of S. senftenberg if such a scheme was needed or desired.

Effect of Inactivation Temperatures of 55°C and 60°C on the Phage Donor Strain SS775W and the Recipient Strain SS32

The hypothesis underlying this phase of the investigation was that the property of "high-level" heat resistance exhibited by the SS775W and SSD8 of Salmonella senftenberg was a genetic trait that could be transferred to susceptible strains of the same species by either the mechanism of phage-mediated transduction or conversion.

To test this hypothesis, cell populations of a heat-sensitive, non-lysogenic strain of Salmonella senftenberg (strain SS32) were exposed to infection with PSS775W phage particles and subjected to continuous cycles of growth (25°C, 35°C) and heating (55°C, 60°C) to select isolates having a transferred genetic determinant for heat resistance. Populations of SS32 cells not exposed to phage were processed in a similar manner to serve as controls.

During the heating phases of the various cycles, survivors were recovered and examined for the properties of lysogeny and heat resistance. Increases in thermal resistance were determined by heating cell-broth suspensions of the individual

isolates and an uncycled (reference) culture of SS32 at 60°C for 2.5 minutes and comparing the survivor levels (percent survivor values or graded colony development patterns) obtained for the isolate with that of the uncycled control. RIPS values were used to express the relative increase in the percent survivor level of the individual test isolate over the %S value determined for the reference culture. Increases in the property of heat resistance were also determined by comparing the thermal inactivation rates and general shapes of the survivor curves plotted for the heating phases of the various cycles. Temperatures of 35°C and 45°C were employed to recover survivors from the heating phases of the various cycles and temperatures of 25°C, 35°C, and 45°C were used to recover survivors in the "60°C - 2.5 minute" screening test for thermal resistance.

The results obtained in this investigation clearly indicate that the effect of heating cell populations of Salmonella senftenberg at temperatures of 55°C and 60°C is more than just the immediate total inactivation of a number of heat-sensitive cells surviving in a population at any given moment. There is strong evidence that these temperatures, in addition to being lethal, are also sublethal for the phage donor strain SS775W and the recipient strain SS32 in the sense that they produced "injured" cells in the survivor population. For the recipient strain, these temperatures were also found to be mutagenic; they produced both high- and low-level "gain" mutations in heat resistance in the uninfected cells and

"cured" some of the infected strains of their lysogens. The results supporting the mutagenic effect of these temperatures are discussed below and are presented at this point to provide information needed to interpret the results obtained in the studies carried out to determine if the character of heat resistance can be genetically transferred in strains of Salmonella senftenberg by bacteriophage and be responsible for the occasional emergence of heat-resistant strains of this species.

Evidence for the Mutagenic Action of Heat on the Phage Recipient Strain SS32. Evidence obtained in this study for the mutagenic action of heat on the phage recipient strain SS32 includes the following:

a. Loss in the ability of heated SS32 cells to grow at 45°C.

b. Gain in the ability of SS331W, a non-lysogenic mutant of SS32 recovered in this study, to grow at 45°C and resist inactivation at a sublethal temperature of 60°C.

c. Decreases in the thermal inactivation rates of SS32 control cell populations in repeated cycles of "growth and heating."

d. Low level increases in thermal resistance in "control" isolates recovered from the heating phases of the "growth and heating" cycles.

e. Prophage induction in SS32 lysogens.

The ability of heated cells to recover at 25"C and 35°C but not at 45°C on nutritionally complex medium can be

interpreted as evidence for the lethal mutagenic effect of heat on the genetic material of thermal-sensitive strains of Salmonella senftenberg. Horowitz and Leupold (1951) isolated temperature-sensitive (ts^+) mutants of E. coli that grew on minimal medium at 25°C but not at 42°C. Most of these mutants had the ability to form colonies on complete medium at 42°C indicating that some substance was required for growth at the higher (restrictive) temperatures but some were able to grow only at the lower (permissive) temperature. These investigators interpreted the inability of the prototrophic mutants to grow at the restrictive temperature as evidence that mutations had occurred in genes that controlled indispensible functions in the cell.

Stent (1971) offers a plausible explanation for the two, temperature-sensitive, mutant phenotypes of E. coli at the molecular level which is based on the influence of environmental temperature on the "particular" higher-level structures finally assumed by polypeptide chain(s) of a protein having an altered and unaltered primary protein structure. He states that "a given protein takes on its biologically functional tertiary and quaternary structure only within a limited 'physiological' temperature range and takes on a nonfunctional or denatured structure outside that range." Thus, the "ts" mutants that can grow in complete medium at 25°C and 42°C are thought by Stent to have "normal" polypeptide chains that allow them to form functional, high-level, protein structures

in this broad temperature range while the "ts" mutants that can only grow at 25°C are thought to have mutant polypeptide chains that will form functional, high-level, structures only at the lower, or permissive, temperature. According to this hypothesis, therefore, loss of the ability to grow at a higher temperature is evidence for damage to the genetic material of the cell.

While loss in the ability of heated SS32 cells to grow at a temperature of 45°C can be considered as a "loss-of-function" or "lethal" mutation, a gain in the ability of heated SS32 cells to grow at this temperature and resist heating at sublethal temperatures of 55°C and 60°C can be considered as a "change-in-function" or "gain" mutation. The heat-resistant strain of SS32 isolated in this study, strain SS331W, displayed this type of mutation. For a "gain" mutation to occur (see Stent, 1971) the primary structures of the gene-controlled, mutant, polypeptide chains of these cells must be altered in such a way that the higher structure of this protein still maintains its indispensable function yet is more resistant to attack by a mutagenic agent than the polypeptide chains of the "wild-type." Because only a very limited number of changes in the primary structures of the polypeptide chains could permit such a change in function, the frequency of "gain" mutations is much lower than that of "lethal" mutations. When the lethal-type mutation occurs, assembly of the polypeptide chains into higher protein structures is prevented regardless of the presence of required growth factors in the recovery medium.

Decreases in the thermal inactivation rates of the SS32 control cell populations during repeated cycles of growth at 35°C and heating at 55°C or 60°C was taken as evidence that heat, acting as a mutagenic agent, was inducing some heat-sensitive cells to become heat-resistant. This was more evident in the second HRT experiment where the cell populations were subjected to an inactivation temperature of 55°C. Supporting this interpretation is the fact that the cells inactivated in the heating phases of the three cycles shown in Figure 8 were the progeny of the same cell population from which the heat-resistant SS331W strain was isolated.

The effect of "cyclic" growth (35°C) and heating (55°C) on the control "B" cells in the second HRT experiment can be seen in a comparison of the thermal inactivation curves for the control cells in cycles II and III with those in cycle I (Figure 8). The progressively decreasing slopes of these curves indicate that the control cells had increased in heat resistance and, in the second cycle, even displayed greater heat resistance than the cells exposed to phage. The D_{55} value calculated for these cells in the first cycle was 0.31 minute and this value increased 6.2-fold in the second cycle and 9.6-fold in the third cycle. The D_{55} value for the PBM cells in the second cycle was 0.74 minute. In the first HRT experiment the control cells grown at 35°C and heated at 60°C had a calculated D_{60} value of 0.46 minute during the first 2.5 minutes of heating in the first cycle and a value of 0.55 minute for the same time period in the second cycle (see 35B curve, Fig. 6).

Further evidence that a temperature of 60°C can be mutagenic for SS32 appears in Table XXII. The results in this table indicate that the "unheated" control cells (the "B: I-55/35-0" sample), which were prepared for the second HRT experiment and subsequently subjected to what can be considered as repeated "cyclic" growth (35°C) and heating at 60°C after prolonged storage at 4°C, also exhibited an increase in heat resistance above that of the SS32 reference culture.

Although a sufficient number of control "B" isolates were recovered in this study (Tables VI, VII, and VIII) to statistically determine whether or not these isolates also showed signs of increased heat resistance, only 8 "B" isolates were "screen" tested for heat resistance. Of this total, 5 were obtained in the third cycle of the second HRT experiment and 3 from the fourth experiment. Two of the isolates in the fourth experiment were later shown to be contaminants. RIPS values calculated from survivor counts at 5 days (Table XIII) indicated that three of the five "third cycle" isolates (B190, B191, and B193) had increased their heat resistance 1.5 to 2.75 fold above that of the uncycled reference strain. These increases, however, were lower than increases in heat resistance observed for SS32 cells lysogenized with PSS775W prophage (see Table XXI) and tested at the same time.

The ability of heat to induce prophage as well as heat-resistant mutants in the recipient strain is indicated in the results of the first HRT experiment where almost all of the 35PBM isolates recovered in Cycle I were nonlysogenic (ly^-)

and where all of the 25 PBM isolates were lysogenic (ly^+) in Cycle I but ly^- in Cycle II. Apparently, prophage is more susceptible to induction at the higher sublethal temperature of 60°C since ly^- isolates were not recovered in the second HRT experiment where the lower temperature of 55°C was employed for inactivation.

Origin of Thermal Resistance in Bacteria: Spontaneous Versus Heat-Induced Mutations. The results under discussion have lead to the conclusion that sublethal temperatures can produce mutations in susceptible, heat-sensitive cells and induce them to become heat-resistant. Such a conclusion violates one of the dogmas of molecular genetics; namely, the spontaneous origin of resistant bacterial variants. According to this tenet, bacterial variants exhibiting resistance to an agent are not induced by treatment with the agent itself but arise spontaneously before treatment and are merely selected by the agent (see Jacob and Wollman, 1961). This theory was precipitated by the classical work of Luria and Delbrück (1943) who demonstrated by a statistical "fluctuation test" that phage-resistant mutants of E. coli arose spontaneously and were not induced by infection with the phage.

Anyone opposed to this dogma fears being branded as a LaMarckist (Luria, 1947) and this fear has influenced the expressed interpretative reasoning of many investigators to the point where they apparently completely ignore evidence suggesting the mutagenic action of heat and consider temperature in relation to mutagenesis only as an environmental factor that

can somehow influence the spontaneous rate of mutation. Fortunately, a few individuals have voiced an opinion or interpreted their experimental findings to the contrary.

David et al. (1968) discuss the origin of spontaneous mutations in bacteria from the viewpoint that such mutations arise as a result of mutagens present in the environment. They consider the correlation between temperature and the spontaneous mutation rate not as evidence that temperature accelerates the action of these mutagens but as an indication that heat itself acts as a mutagen. They speculate that heat may cause occasional breakage of a thermally activated bond in a nucleotide of DNA or cause ambiguity of base pairing. Another possibility is that this agent may cause illegitimate base pairing of the rare imino form of adenine with cytosine and the rare enol form of thymine with quanine (see Stent, (1971).

The mutagenic effect of heat was reported by Zamenhof and Greer (1958) who produced nutritional (thy^-, Pu^-) and fermentative (lac^-, mal^-, and glu^-) mutants of E. coli by heating the cells at "sub-lethal" temperatures of 55°C and 60°C in the dried state, in broth, and in 50 percent sucrose solutions. The strains of E. coli used in their study were "mutants" that had survivor level values (%S values) higher than other strains under the conditions of growth and testing. Zamenhof (1960) later reported that much higher "mutant frequencies," i.e., mutation rates, could be obtained for E. coli and Bacillus subtilis if these organisms were heated at 135°C and 155°C

in the dried state under vacuum for 16 minutes. Zamenhof's argument that his auxotrophic mutants were heat-induced and not selected for was that these mutants were not reselected in much higher proportions when they were equally mixed with the parent prototroph and heated and recovered under the same experimental conditions. This argument supported the obvious fact that an abnormally high proportion of mutant types were recovered in his experiments.

In conjugation experiments with E. coli (Zamenhof and Greer, 1958) and in transformation experiments with B. subtilis (Zamenhof, 1960), the nutritional mutants obtained as a result of heating were demonstrated to segregate their "heat-induced" markers indicating that the carrier of the injured nutritional determinant was DNA and that the mutation was chromosomal and not cytoplasmic in nature. Zamenhof and his co-workers (Chiasson and Zamenhof, 1966; Greer and Zamenhof, 1962) postulated that at high temperatures heat acts at random on the genetic material to remove purine bases (depurination).

Heat has also been reported to have a mutagenic effect on sporulation in B. subtilis. Northrop and Slepecky (1967) found that heating spores of this organism at 90°C and 100°C in the dried state under vacuum produced not only auxotrophic mutants but sporulation mutants which exhibited differences in cellular and colonial morphology on germination as well as differences in pigmentation on Potato Dextrose agar. These authors agreed with Zamenhof's theory of depurination of the

genetic material at the higher processing temperature (100°C) but suggested the possibility that the high mutant frequencies and the low death rate for spores at the lower temperature (90°C) might also be due to damage and destruction of susceptible cytoplasmic factors.

As stated, many workers are reticent to be identified with a concluding statement that heat acts as a mutagenic agent on vegetative bacteria at sublethal temperatures. Allwood and Russell (1970), for example, mention the work of Chiasson and Zamenhof (1966) and Norton and Slepecky (1967) on the production of dry heat-induced mutations in spores of B. subtilis at high temperatures but make no reference to the earlier studies of Zamenhof and Greer (1958) in which these workers offered evidence that heat-induced mutations were produced in the genetic material of E. coli at temperatures of 55°C and 60°C. With this omission, the reviewers proceeded to gather sufficient information from other sources (Jordon et al., 1947; Jackson and Woodbine, 1963; and Harries, 1968) to make a summary remark that "there is no evidence available at present implicating the induction by heat of stable mutants of vegetative bacteria."

Allen (1953) describes a procedure generally used for the isolation of thermophilic variants of Bacillus species from growing cultures of mesophiles that produce mutation frequencies to thermophily (ability of an organism to grow at 55°C) of about 10^{-6}. In this procedure an inoculum from a "primary" culture of a mesophilic organism is first transferred to a

tube of liquid medium and incubated at 35°C - 40°C until a suitable growth is obtained. The culture is then diluted with fresh medium and heated (incubated) at 55°C for several hours. After this initial period of incubation, a portion of the culture is transferred to a fresh tube of medium and incubation is continued at the elevated temperature. When the culture shows evidence of good growth it is streaked to solid medium for isolation of thermophilic variants. As a negative control, the "primary" mesophilic culture is tested for its inability to grow at 55°C. Thermophilic variants have been produced from spore suspensions of mesophiles using the same protocol.

While the procedure described by Allen for the isolation of thermophilic variants of Bacillus species is different from the one employed in this study to isolate heat-resistant variants of S. senftenberg, the end result and the general method used to obtain this result were the same; in both cases, thermal variants were recovered by heating cultures of the organisms at temperatures higher than their "normal" maximum limit for growth. The underlying question regarding the origin of thermophilic variants is whether they are produced or selected by heat. Allen's opinion to this effect is put forth in a carefully worded statement--"Until such time as a mutagenic action of heat is clearly demonstrated, it appears preferable to consider the outcome of the experiments as the selection of spontaneously occurring mutants, rather than the production of resistant individuals by the action of heat." The

"spontaneous mutation" hypothesis would be more believable if the controls, which were exposed to the same elevated temperature, had been positive instead of negative.

Allen's review was published prior to the reported studies of Norton and Slepecky and Zamenhof and his co-workers and knowledge of their findings and conclusions may have changed her statement regarding the origin of thermophilic variants. One, however, is surprised to find a condensed version of her statement appearing as a summary remark in a recent review on "Inhibition and Destruction of Microorganisms by Heat (Brown and Melling, 1971)" written 18 years later--"Observations on some mesophilic bacteria (Mefferd and Campbell, 1952; Allen, 1953) have indicated the presence of about one thermophilic bacteria variant in 10^6 cells of the original population. Thus, in the absence of evidence of any mutagenic action of heat, it appears that these thermophiles may have arisen by spontaneous mutation."

Conversion of a Heat-Sensitive Strain of S. senftenberg (Strain 32) to "Low-Level" Heat Resistance by S. senftenberg 775W Bacteriophage

The decisions to be made regarding the results obtained in the HRT experiments is whether or not genetic determinants for heat resistance (hr) were transferred by SS775W phage particles from the heat-resistant donor strain SS775W to the heat-sensitive recipient strain SS32, and whether the noted increase in heat resistance was due to viral genes (phage-mediated conversion) or bacterial genes (phage-mediated transduction) carried by the phage particles.

The thermal inactivation curves plotted from the survivor data obtained in the first and second HRT experiments (Figures 6 and 8) indicated that SS32 cells exposed to SS775W phage particles (PBM cells) were more heat-resistant than the control (B) SS32 cells that were not exposed to phage. The PBM cells displayed greater thermal resistance than the B cells in the heating phases of both cycles of the first HRT experiment (Figure 6) and in the first and third cycles of the second HRT experiment (Figure 8). These curves also revealed that control cells which were grown at 35°C and survived inactivation at 55°C and 60°C in the heating phases of the first cycle exhibited increases in heat resistance in subsequent cycles. The mutagenic effect of heat on the control cells was most noticeable for Curve B in the second cycle of HRT experiment No. 2 (Figure 8). Because increases in heat resistance in the control cell populations did not appear until after the first cycle, increases in heat resistance for the PBM cells during this cycle were taken as evidence that genetic determinants for "low-level" heat resistance had been transferred to the recipient cells by the phage particles.

The weight of experimental evidence in this study indicates that the genetic determinant for heat resistance carried by the SS775W phage particles were viral rather than bacterial in origin. This singles out phage-mediated conversion as the mechanism for genetic transfer.

Much of the evidence for phage conversion comes from the early phases of thermal inactivation at 60°C and 55°C in the

first cycles of the first two HRT experiments (Figures 6 and 8) where all the cells were lysogenic prior to heating (see PBM curve, Cycle I in Figure 7). During the early phases of heating the approximate linear inactivation rates for the ly^+ cells were less than the ly^- control cells and at the lower temperature of inactivation "multiple hits" were required to inactivate the ly^+ cells before linear inactivation was achieved.

The appearance of decreased inactivation rates and a "multiple-hit" requirement during the early stages of "first cycle" thermal inactivation at 55°C argues against transduction being the chief mechanism for genetic transfer. With a transduction frequency of ca 10^{-5} to 10^{-6} (Iino and Lederberg, 1964) for a genetic determinant for heat resistance we would expect the transductants to appear at very low survivor levels with the resultant curve being of the "exponential-convex" type with an inactivation rate during the exponential phase equal to that of the control.

Of the 115 isolates recovered from the heating phases of the various cycles and tested for the properties of thermal resistance and lysogeny, 104 were PBM isolates and 11 were B isolates. Five of the PBM and two of the B isolates were demonstrated to be contaminants. Of the 99 "true" PBM isolates, two were hr^+,ly^-, seven hr^-,ly^-, 45 hr^+,ly^+, and 45 hr^-,ly^+. Of the nine "true" B isolates three were hr^+,ly^- and six hr^-,ly^-.

The results of tests for heat resistance and lysogeny performed on the isolates showed that either one or two "hr, ly" phenotypes were recovered in each cycle of "growth and heating." With the exception of nine of the PBM isolates in the first HRT experiment which were hr^+,ly^- and hr^-,ly^- (the 35 PBM isolates in Cycle I and the 25PBM isolates in Cycle II) all the PBM isolates recovered and tested in HRT experiments Nos. 1, 2, and 4 were either hr^+,ly^+ or hr^-,ly^+. The test results also showed that the B isolates recovered in Cycle I of the fourth HRT experiment and in Cycle II of the second HRT experiment were hr^-,ly^- and that those recovered in Cycle III of the second HRT experiment were hr^+ly^- and hr^-,ly^-.

Normally, isolation of survivors with hr^+,ly^- phenotypes in experiments designed to transduce genetic determinants for heat resistance would be sufficient justification to take a position that the isolate was an "hr" transductant. With foreknowledge that the inactivation temperatures of 60°C and 55°C acted as mutagenic agents which induced heat resistance in the recipient cell such a position is less tenable. As a result, isolates with hr^+,ly^- phenotypes in this study were considered to be heat-induced mutants.

It was hoped that the results of these tests would unquestionably support the interpretation made of the plotted survivor data; namely, that the increase in heat resistance exhibited by the SS32 recipient cells after exposure to SS775W

phage particles was due to infection of these cells with prophage from a normal virion. For this to happen, the PBM isolates recovered in the heating phases of the various cycles should display either the hr^+,ly^+ or the hr^-,ly^- phenotype indicating that the increase in heat resistance is prophage dependent. Contrary to what was expected, ca 90 percent of the PBM isolates were either hr^+,ly^+ or hr^-,ly^+. Both of these phenotypes were recovered in most of the cycles but in different proportions (1:1, 2:1, 3:1, and 19:1).

The recovery of a relatively large number of PBM isolates with hr^-,ly^+ characteristics indicates either that phage conversion did not actually occur or that the portion of the viral genome determining the increase in heat resistance was present, but for some reason, was not being expressed. In the light of strong evidence for increased heat resistance in the population of cells that were exposed to and infected with SS775W phage, the "restricted-phenotypic expression" hypothesis has more appeal and is accepted rationale according to the "operon" theory proposed for the synthesis and regulation of enzymes associated with lactose utilization in Escherichia coli (Jacob and Monod, 1961). In brief, this theory states that "genes encoding for the structure of enzymes of related function are assembled as operons on the bacterial chromosome, so that their expression can be controlled coordinately to the needs of the cell through interaction of a repressor with a common operator gene (Stent, 1971)."

If the differences in the phenotypes of the PBM isolates are explainable in terms of the "operon" concept then the hr^-, ly^+ isolates would be said to contain a viral structural gene (hr^+) which has the ability to form a protein product that can, in some way, increase the heat resistance of the cell but is prevented from expressing this ability because it is in a state of interaction with a substance called a repressor. This repressor, a diffusible product of the regulator or repressor gene, complexes with the operon at a complementary site called the operator gene and, in so doing, prevents the transcription of the structural gene's polygeneic coded message into messenger RNA (mRNA) which must attach to the surface of the cell's ribosomes before the specific protein product of the gene can be synthesized. The function of the ribosomes in protein synthesis is to orient the growing polypeptide chain and amino acid precursors in a suitable stereochemical position with mRNA so that the message coded in mRNA can be accurately read. For this orientation, the amino acid precursors are preattached to soluble RNA (sRNA) or transfer RNA (tRNA) adaptor molecules by means of high energy bonds (see Watson, 1965). Additional studies on the lac operon have shown that the repressor substance is a protein (Gilbert and Müller-Hill, 1967) and that the genetic site at which the RNA polymerase enzyme first attaches to the structural gene to begin transcription of mRNA is not the same interaction site of the repressor. Jacob et al. (1964) have called this

site the promoter gene whose chief function is to determine the frequency of attachment of the RNA polymerase enzyme which, in turn, will set the maximum rate at which the gene can transcribe its message, even in the event that the operator gene is being fully derepressed.

Prophage genes, with the notable exception of the immunity repressor gene which prevents multiplication of the prophage and any superinfecting phage, are considered to be under repression in the infected host cell (Luria and Darnell, 1967). There are, however, some known instances in which these genes are not repressed and their products are used in the metabolism of the host bacterium to form a cellular component that is quantitatively and/or qualitatively different from that of the "normal" host. A case in point is that of the antigenic conversion in the salmonellae where, for example, Salmonella phage ε^{34} can convert Sub-group E_2 strains of Salmonella (O antigens 3, 15) into Sub-group E_3 (O antigens 3, 15, 34) by reportedly adding glucose to the terminal galactose sugar (Uchida et al., 1963).

To explain the hr^+, ly^+ characteristics of PBM isolates in terms of the "operon" theory, we would say that the structural gene of the prophage that carries the determinant for heat resistance is not being repressed and is, therefore, able to express itself and alter or interfere with the cell's normal character of temperature tolerance. This could be brought about by (a) a mutation in the operator gene so that its complementary site can no longer complex with the

repressor substance, (b) a mutation in the regulator gene which causes the production of an inactive repressor substance which cannot complex with the operator gene, or (c) the formation of a complex between the repressor and a co-repressor substance (a metabolite or its analogue) which serves to inactivate the repressor and allow the structural gene to be expressed.

Bacteriophages have been reported to contain heat-sensitive and heat-stable immunity repressor genes. Goldwaith and Jacob (1964) isolated a mutant of E. coli lysogenized with phage lambda in which the prophage could be induced at 42°C but not at 30°C. Lysis of bacteria at the higher temperature was thought to be due to a mutation in the immunity repressor gene which caused it to produce a temperature-sensitive repressor (see Luria and Darnell, 1967). Lieb (1966) showed that the bacteria containing these mutant phages (λC_I^{ts}) could be protected from lysis at 42°C if they were superinfected with a "wild type" phage (λC_I^{+}) which acted to supply a heat-stable repressor. The mechanism of prophage induction is still not completely understood, however, and there is evidence to cause some to believe (see Luria and Darnell, 1967; Hayes, 1968) that the immunity repressor is not inactivated because it is labile to heat but because it complexes with co-repressors. These co-repressors are considered to be DNA precursors which accumulate when DNA synthesis is interrupted by UV light and other agents with similar action.

Origin and Nature of Thermal Resistance in Strains of Salmonella senftenberg Possessing This Property

The study being reported was fundamentally based on two hypotheses: (1) that the reported heat-resistant strains of Salmonella senftenberg, the SS775W and SSD8 strains, carried temperate bacteriophages and (2) that the property of "high-level" heat resistance exhibited by these two strains could be transferred to susceptible strains of the same species by either the mechanism of phage-mediated conversion or transduction. The experimental findings indicated that the heat-resistant strains carried related phages and that one of the phage-indicator strains employed for their detection was both heat-sensitive and nonlysogenic and could, therefore, act as a recipient strain for the genetic material carried by the phage particles. To demonstrate phage-mediated transfer of a genetic determinant for "high-level" thermal resistance, SS775W was used as the phage donor strain and strain SS32 as the recipient. The results of this study indicated that the phage lysates contained no transducing particles carrying genetic determinants for "high-level" thermal resistance but that the prophage of SS775W did carry a genetic determinant that could convert the recipient strain to a low-level of heat resistance.

In the course of this study it was also found that temperatures of 55°C and 60°C employed for inactivation of cell populations of SS32 and SS32 lysogenized with prophage of SS775W had a sublethal as well as lethal effect on the cells

in these populations. Part of the sublethal effect was shown to be mutagenic since heating at these temperatures was shown to "cure" lysogenized cells of their prophage, cause cells to lose their ability to grow at 45°C, and induce them to a low- and high-level of thermal resistance.

Based on these findings, the second hypothesis upon which this study was based was rejected in favor of the hypothesis that strains of S. senftenberg with "high-level" heat resistance originated either by spontaneous mutation or a mutation induced by some chemical agent or a physical agent such as heat. Of the three known strains of S. senftenberg reported to have high-levels of heat resistance (SS775W, SSD8, and now SS331W), two have had a previous history of exposure to sublethal temperatures before isolation--the SS775W strain and the SS331W strain which is an hr^+ mutant of SS32.

To be added to the already mounting evidence that the primary cause of death in non-sporulating, mesophilic bacteria is degradation of the ribosomes and their nucleic acid (see Allwood and Russell, 1970; Campbell and Pace, 1968) is the finding of Ozaki et al. (1969) that the streptomycin (strA) locus in E. coli codes for a single protein in the 30S particle of the ribosome and the reports of other investigators (McDonald and Mattney, 1963; Tai et al., 1969; and Kang, 1970) which indicate there is a gene closely associated with this locus that controls the thermostability of this protein. When these findings are totaled and combined with (1) a knowledge of the differences between the streptomycin locus of E. coli

and S. typhimurium and (2) an awareness of the "corrected" fundamental principle of molecular biology which states that the primary structure of polypeptide subunits determines the higher-level structures of proteins "under normal environmental conditions," then some insight can be gained into the nature of "high-level" thermal resistance in strains of Salmonella senftenberg possessing this property.

There is, therefore, some evidence on which to construct a theory that the str protein, i.e., the protein product of the str locus altered by streptomycin, is also the primary site in the bacterial cell that determines its sensitivity and resistance to heat as well as its ability to grow at high and low temperatures. Depending on the amount of thermostability it possesses, one can visualize this protein in a bacterial cell being subjected to thermal stress and undergoing various degrees of denaturation, i.e., the separation of its polypeptide subunits and the unfolding and uncoiling of its individual chains as secondary heat-sensitive bonds are being broken in the higher-level structures. As denaturation proceeds there is eventually a point where the molecules of this protein, its associated proteins, and rRNA cease to interact and the nucleic acid is degraded by enzymes or denatured by heat. Denaturation of rRNA by heat is mentioned because Campbell and Pace (1968) have shown that the melting point temperature of rRNA is lower than either the maximum growth temperature of the organism or the melting point of the ribosome. This finding suggests that rRNA is more heat stable

in the combined (protein-interacting) state than in the free state in which it is not interacting with protein.

With their release from thermal stress and their return to optimum growth conditions, bacterial cells can repair the damage done to their protein synthesizing machinery by resynthesizing new rRNA molecules and reconstituting them with their associated proteins into new ribosomes; providing, of course, that other lethal events have not occurred in the cell and the encoding genes for the ribosomal proteins, especially the str protein, have not been altered. Cell populations that have been exposed to sublethal temperatures and require incubation periods significantly longer than unheated cells to form their maximum number of colonies are considered to be cells that have received thermal injury.

As part of this study, cell populations of a heat-sensitive (hr^-) strain of S. senftenberg (SS32) and a heat-resistant (hr^+) strain (SS775W) were tested for their ability to recover on complete medium at incubation temperatures of 25°C, 35°C, and 45°C after being heated in broth medium at 60°C for 2.5 minutes. Unheated cell populations of both strains, serving as controls, had the ability to grow at all three temperatures and form their maximum number of colonies in 24 hours at 35°C and 45°C and in 48 hours at 25°C. It was found that survivors in the heat-treated populations of hr^- cells failed to grow at 45°C and recovered much slower at 25°C and 35°C than either the unheated control cells or the heat-treated

cells of the hr^+ strain which recovered more rapidly at 35°C and 45°C than at 25°C and were only slightly inhibited from growing at 45°C. Heat-treated survivors and the unheated cells of both strains grew more rapidly at 35°C than at either of the other two temperatures.

Interpreting these results in terms of the str protein and its encoding gene, these findings indicate that the str protein of the hr^- strain is more easily denatured by heat than the corresponding protein of the hr^+ strain. The ability of the str protein of the hr^+ strain to resist thermal denaturation must reside in the gene-controlled, primary structures of its polypeptide subunits which are able to impart this characteristic property to the protein as it proceeds to coil, fold, and unite by secondary bonds to form the higher-level structures which interact with rRNA and other ribosomal proteins to form the 30S particle of the ribosome.

Heat induction studies by Mefferd and Campbell (1952) and the study being reported indicate that the gene regulating the thermostability of the str protein can be altered by sublethal temperature. Almost half (46.3%) of the 108 "true" isolates recovered in this study during the various heating phases of repeated "growth and heating" cycles, which was the method used in this study to select heat-resistant mutants from mixed suspensions of phage and bacteria (PBM suspensions) and survivors from control (B) suspensions, showed low-level increases in heat resistance above that determined for the uncycled SS32 reference strain. Only the hr^+ mutant of SS32, the SS331W

strain, exhibited "high-level" thermal resistance comparable to that of the two reported heat-resistant strains of S. senftenberg (SS775W and SSD8).

Identification of a genetic determinant regulating the thermostability of the protein product of the str locus in various bacteria offers a possible explanation as to how the genetic material of S. senftenberg can be altered to produce mutants with either a high- or low-level of heat resistance. In attempting such an explanation it must first be assumed that the genetic determinants for streptomycin resistance in S. senftenberg are the same as those for S. typhimurium. Sanderson's "138-minute" circular linkage map for the chromosome of Salmonella typhimurium (Sanderson, 1967) has been compiled from the reported results of interrupted conjugation and transduction experiments and shows two loci for streptomycin resistance--the strA locus and strB locus. The strA locus, which is responsible for "high-level" resistance to streptomycin, is positioned on the map at 108 minutes and the strB locus, which is responsible for "low-level" resistance to streptomycin and also the character of auxotrophy, is located at the 80-minute map position. Only a single locus for streptomycin resistance (the strA locus) has been demonstrated for E. coli (see Sanderson, 1967). If the str loci demonstrated to be present in S. typhimurium are also present with similar functions in S. senftenberg, then it is possible that the low-level increases in heat resistance observed in the isolates recovered in this study can be attributed to mutations occurring

in the gene regulating the thermostability of the strB locus and the high-level increase observed for SS331W, the hr^+ mutant of SS32, to a mutation occurring in the corresponding gene to the strA locus.

Mutations in the strA locus of E. coli are constantly being suppressed by genes at the ram locus, i.e., "amber-type" genes that suppress "non-sense" mutations affecting the ability of the ribosomes to faithfully translate the polygeneic coded message carried by the RNA messenger. These suppressor genes map in the 30S ribosomal cluster between strA (64 minutes) and aroE which is located at the same time position on the map of E. coli (Rossett and Gorini, 1969). The corresponding regional location for this locus on the chromosomal map of S. typhimurium would be between strA (108 minutes) and aroC (120 minutes). Nomura et al. (1969) speculate that the ram locus codes for the P7 protein isolated by Ozaki et al. (1969) which these workers found to be required for decreasing ambiguity of translation in ribosomes that were reconstituted with 30S particles deficient in known proteins.

To produce a strain of S. senftenberg having a high-level of heat resistance, it is most likely that a mutation(s) must occur in a suppressor gene(s) at the ram locus as well as in the temperature-regulating gene at the strA locus. If this is true, it lessens the possibility that strains possessing this unique characteristic arise spontaneously and strengthens the argument that the most probable origin of heat-resistant strains of Salmonella senftenberg is heat itself.

Significance of Study in Relation to Public Health

The results of this study indicate that inactivation temperatures of 55°C and 60°C have a sublethal as well as a lethal effect on nonsporing bacteria. Heating at these temperatures can cause alterations in genes which will give rise to heat-resistant mutants and can produce injury to gene products which the cell must repair before it can resume growth and division. It is suggested from this study (1) that regulatory agencies responsible for recommending minimum "time-temperature" requirements for cooking base their recommendations on total destruction of pathogens commonly associated with particular foods and food products to avoid the risk of inducing heat-resistant mutants, and (2) that incubation times for recovery of pathogens from cooked foods and food products be extended to allow for recovery of heat-injured survivors that are frequently present in low numbers but remain undetected by procedures often employed for their isolation.

CONCLUSIONS

The results obtained in this study lead to the general conclusion that the high-level of heat resistance exhibited by the Winter (775W) and Davidson (D8) strains of Salmonella senftenberg is genetic in origin and can be attributed to mutations produced by heat rather than to genes acquired by the transfer mechanisms of phage-mediated conversion or transduction. Specifically, the results indicate that

1. Most, if not all, strains of Salmonella senftenberg are lysogenic and are, therefore, amenable to phage typing.
2. The heat-resistant strains of S. senftenberg, the Winter and Davidson strains, carry related prophages.
3. S. senftenberg 775W prophage is inducible with ultraviolet irradiation.
4. S. senftenberg 775W phage (PSS775W) requires divalent cation for host attachment, is stimulated by increased concentrations of magnesium ion, and is moderately sensitive to chloroform and not readily adsorbed onto cellulose triacetate filter membranes.
5. Attachment of PSS775W to a host cell can be prevented with citrate ion which acts as a chelating agent.

6. S. senftenberg strain 32, a recipient host strain for PSS775W, is nonlysogenic and heat-sensitive.
7. Susceptible strains of S. senftenberg can be converted to a low-level of heat resistance by S. senftenberg 775W phage particles but cannot be converted or transduced to a high-level of heat resistance.
8. Viral genetic determinants for "low-level" heat resistance may be repressed in the recipient host cell.
9. Elevated temperatures of 55°C and 60°C have a sublethal as well as lethal effect on lysogenized and nonlysogenized cells of S. senftenberg; heating at 55°C or 60°C can cause injury to gene products and heating at 60°C can produce mutations in genes.
10. S. senftenberg strain 331W is a heat-induced, heat-resistant mutant of S. senftenberg strain 32 whose thermal resistance is comparable to that of the Winter and Davidson strains. This strain can be differentiated from the 775W and D8 strains of S. senftenberg and identified with strain 32 by biochemical tests and tests to determine its immunity or susceptibility to phages produced by the heat-resistant strains.

LITERATURE CITED

Adams, M. H. 1959. Bacteriophages, 1st ed., pp. 56-60, 450-451. Interscience Publishers, New York.

Algranati, I. D. and P. Lengyel. 1966. Polynucleotide-dependent incorporation of amino acids in a cell-free system from thermophilic bacteria. J. Biol. Chem. 241:1778-1783.

Allen, M. B. 1953. Thermophilic aerobic sporeforming bacteria. Bacteriol. Rev. 17:125-173.

Alloway, J. L. 1932. The transformation in vitro of R pneumococci into S forms of different specific types by the use of filtered pneumococcus extracts. J. Exp. Med. 55:91-99.

Allwood, M. C. and A. D. Russell. 1970. Mechanisms of thermal injury in nonsporulating bacteria. Advan. Appl. Microbiol. 12:89-119.

Andrewes, F. W. 1922. Studies in group-agglutination. I. The Salmonella group and its antigenic structure. J. Pathol. Bacteriol. 25:505-521.

Andrewes, F. W. 1925. Studies in group agglutination. II. The adsorption of agglutinin in the diphasic Salmonellas. J. Pathol. Bacteriol. 28:345-349.

Anellis, A., J. Lubas, and M. M. Rayman. 1954. Heat resistance in liquid eggs of some strains of the genus Salmonella. Food Res. 19:377-395.

Arber, W. 1960. Transduction of chromosomal genes and episomes in Escherichia coli. Virology 11:273-288.

ARS Report. 1966. The destruction of salmonellae. A Report of the Western Experiment Station Collaborators Conference (Western Regional Research Laboratory, Albany, California, 1966). Western Utilization and Development Division, Agricultural Research Service, U. S. Dept. Agric. ARS Report No. 74-37.

Atkinson, N. 1957. Lysogenicity and lysis patterns in the Salmonellas. 11. The bacteriophage grouping scheme compared with other schemes using bacteriophage for exact strain identification in the Salmonellas. Austral. J. Exp. Biol. Med. Sci. 35:1-14.

Avery, O. T., C. M. MacCleod, and M. McCarthy. 1944. Studies on the chemical nature of the substance inducing transformation of pneumococcal types. Induction of transformation by a desoxyribonucleic acid fraction isolated from Pneumococcus type III. J. Exp. Med. 79: 137-158.

Barksdale, L. 1959. Lysogenic conversions in bacteria. Bacteriol. Rev. 23:202-212.

Baron, L. S., W. M. Spilman, and W. F. Carey. 1959a. Hybridization of Salmonella species by mating with Escherichia coli. Science 130:566-567.

Baron, L. S., W. F. Carey, and W. M. Spilman. 1959b. Characteristics of a high frequency of recombination (Hfr) strain of Salmonella typhosa compatible with Salmonella, Shigella, and Escherichia species. Proc. Nat. Acad. Sci. U.S.A. 45:1752-1756.

Bayne, H. G. 1966. Reexamination of heat resistance of Salmonella typhimurium in ground chicken. In ARS Report, 1966. The destruction of salmonellae. Ibid., pp. 37-39.

Beale, G. H. and J. F. Wilkinson. 1961. Antigenic variation in unicellular organisms. Annu. Rev. Microbiol. 15: 263-296.

Boring, J. R. 1965. Interstate outbreak of Salmonella derby gastroenteritis: The field studies. In NCDC, 1965. Ibid., pp. 122-125.

Bornstein, S. and I. Saphra. 1942. The occurrence of unusual Salmonella species. J. Infect. Dis. 71:55-56.

Brown, M.R.W. and J. Melling. 1971. Inhibition and destruction of microorganisms by heat. In W. B. Hugo (ed.), Inhibition and destruction of the microbial cell, pp. 1-37. Academic Press, Inc., New York.

Campbell, L. L. and B. Pace. 1968. Physiology of growth at high temperatures. J. Appl. Bacteriol. 31:24-35.

Cherry, W. B., M. Scherago, and R. H. Weaver. 1943. The occurrence of Salmonella in retail meat products. Amer. J. Hyg. 37:211-215.

Chiasson, L. P. and S. Zamenhof. 1966. Studies on induction of mutations by heat in spores of Bacillus subtillus. Can. J. Microbiol. 12:43-46.

Clarenburg, A., H. H. Vink, and B. C. Noordhoff. 1950. Infection with Salmonella saint-paul following ingestion of Salmonella-infected duck eggs. Nederl. Tijdschr. Geneesk. 94:1615-1618.

Davidson, C. M. 1967. Personal communication.

Davidson, C. M., M. Boothroyd, and D. L. Georgala. 1966. Thermal resistance of Salmonella senftenberg. Nature (London) 212:1060-1061.

Davis, B. D., R. Dulbecco, H. N. Eisen, H. S. Ginsberg, and W. B. Wood, Jr. 1968. Microbiology, 1st ed., pp. 170-182; 1113-1118; 205-207; 280-281; 1118-1126; 246, 237. Harper and Rowe, New York.

Dawson, M. H. and R.H.P. Sia. 1931. In vitro transformation of pneumococcal types. I. A technique for transformation of pneumococcal types in vitro. J. Exp. Med. 54:681-699.

Demerec, M. and P. E. Hartman. 1959. Complex loci in microorganisms. Annu. Rev. Microbiol. 13:377-406.

Edwards, P. R. 1965. Salmonellosis--an expanding problem. In NCDC, 1965. Ibid., pp. 7-12.

Edwards, P. R. 1939. Incidence of Salmonella types in fowls in the United States. Proc. 7th World Poult. Congr. Expos. (Cleveland), pp. 271-274.

Edwards, P. R. 1937. A new Salmonella type possessing a hitherto undescribed non-specific antigen. J. Hyg. (London), 37:384-387.

Edwards, P. R. and D. W. Bruner. 1943. The occurrence and distribution of Salmonella types in the United States. J. Infect. Dis. 72:58-67.

Edwards, P. R. and D. W. Bruner. 1942. Serological identification of Salmonella cultures. Univ. of Kentucky Agr. Exp. Sta. Circ. 54.

Edwards, P. R. and D. W. Bruner. 1938. Two new Salmonella types isolated from fowls. J. Hyg. (London), 38: 716-720.

Edwards, P. R., D. W. Bruner, and A. B. Moran. 1948. Further studies on the occurrence and distribution of Salmonella types in the United States. J. Infect. Dis. 83:220-231.

Edwards, P. R. and W. H. Ewing. 1962. Identification of Enterobacteriaceae, 2nd ed. Burgess Publishing Co., Minneapolis.

Edwards, P. R., A. C. McWhorter, and M. A. Fife. 1954. Studies on group G of the genus Salmonella. J. Bacteriol. 67:346-349.

Edwards, P. R., A. B. Moran, and D. W. Bruner. 1947. Intertransformability of Salmonella simsbury and Salmonella senftenberg. Proc. Soc. Exp. Biol. Med. 66:230-232.

Eijkman, C. 1908. Die Ueberlebungskurve bei Abötung von Bakterien durch Hitze. Biochem. Z. 11:12-20.

Falkow, S., R. Rownd, and L. S. Baron. 1962. Genetic homology between Escherichia coli K-12 and Salmonella. J. Bacteriol. 34:1300-1312.

Farrell, J. and A. H. Rose. 1967. Temperature effects on microorganisms. Annu. Rev. Microbiol. 21:101-120.

Fredericq, P. 1965. Genetics of colicinogenic factors. Zentralb. Bakteriol. Parasitenk. Abt. I. Orig. 196:142-151.

Friedman, S. M., R. Axel, and I. B. Weinstein. 1967. Stability of ribosomes and ribosomal nucleic acid from Bacillus stearothermophilus. J. Bacteriol. 93: 1521-1526.

Galbraith, N. S., J. F. Archer, and G. H. Tee. 1961. Salmonella saint-paul infection in England and Wales. J. Hyg. (London), 59:133-141.

Gaughran, E.R.L. 1947. The thermophilic microorganisms. Bacteriol. Rev. 11:189-225.

Gilbert, W. and B. Müller-Hill. 1967. Isolation of the lac repressor. Proc. Nat. Acad. Sci. U.S.A. 58:2415-2421.

Gill, F. A. and E. W. Hook. 1966. Salmonella strains with transferable anti-microbial resistance. J. Amer. Med. Assoc. 198:1267-1269.

Goldthwait, D. and F. Jacob. 1964. Sur le méchanisme de l'induction du développement du prophage chez les bactéries lysogènes. C. R. Acad. Sci. (Paris) 259:661-664.

Greer, S. and S. Zamenhof. 1962. Studies on depurination of DNA by heat. J. Mol. Biol. 4:123-141.

Griffith, F. 1928. The significance of pneumococcal types. J. Hyg. (London) 27:113-159.

Hansen, N.H. and H. Rieman. 1963. Factors affecting the heat resistance of nonsporing organisms. J. Appl. Bacteriol. 26:314-333.

Harada, K. 1959a. Studies on directed variation of Salmonella 034 by bacteriophage. I. Antigenic transformation by bacterial autolysate and antiserum. Japan. J. Microbiol. 3:53-60.

Harada, K. 1959b. Studies on directed variation of Salmonella 034 by bacteriophage. II. Antigen transforming agent in bacteria of group E3. Japan. J. Microbiol. 3:61-69.

Harries, D. 1968. Ph.D. Thesis, University of Wales.

Hartman, P.E. and S. H. Goodgal. 1959. Bacterial genetics (with particular reference to genetic transfer). Annu. Rev. Microbiol. 13:465-504.

Harvey, R.W.S. and T. H. Price. 1962. Salmonella serotypes and Arizona paracolons isolated from Indian crushed bone. Pub. Hlth. Lab. Serv., Monthly Bull. Minist. Hlth. (London) 21:54-57.

Haupt, H. 1932. Der gegenwärtige Stand der Systematik und Benennung der Bakterien und ihre Anwendung in der medizinischen Bakteriologie. Ergebn. Hyg. 13:641-685.

Hayes, W. 1968. The genetics of bacteria and their viruses, 2nd ed., pp. 473-474. John Wiley & Sons, Inc., New York.

Hayes, W. 1964. The genetics of bacteria and their viruses, 1st ed., pp. 547-550; 628-629; 523-550; 377. John Wiley & Sons, Inc., New York.

Hobbs, B. C. 1965. Salmonella in foods. In NCDC, 1965. Ibid., pp. 84-94.

Hormaeche, E., C. A. Peluffo, and P. L. Aleppo. 1939. Las salmonelas en patologia infantil. Abst. 3rd Internatl. Congr. Microbiol. (New York), pp. 274-275.

Hormaeche, E., N. L. Surraco, C. L. Peluffo, and P. L. Allepo. 1943. Cause of infantile summer diarrhea. Amer. J. Dis. Child. 66:539-551.

Horowitz, N. H. and V. Leopold. 1951. Some recent studies bearing on the one gene-one enzyme hypothesis. Cold Spring Harbor Symp. Quant. Biol. 16:65-74.

Hussemann, D. L. and J. K. Buyske. 1954. Thermal death time-temperature relationships of Salmonella typhimurium in chicken muscle. Food Res. 19:351-356.

Iandolo, J. J. and Z. J. Ordal. 1966. Repair of thermal injury of Staphylococcus aureus. J. Bacteriol. 91: 134-142.

Iino, T. and J. Lederberg. 1964. Genetics of Salmonella In van Oye, E. (ed.), The world problem of salmonellosis, pp. 111-142. Dr. W. Junk, The Hague, The Netherlands.

Ingraham, J. L. 1962. Temperature relationships. In I. C. Gunsalus and R. Y. Stanier (eds.), The bacteria, vol. 4, pp. 265-298. Academic Press, New York.

Iseki, S. and T. Sakai, 1953. Artificial transformation of O antigen in Salmonella E group. Transformation by antiserum and bacterial autolysate. Proc. Japan. Acad. Sci. 29:121-126.

Jackson, H. and M. Woodbine. 1963. The effect of sublethal heat treatment on the growth of Staphylococcus aureus. J. Appl. Bacteriol. 26:152-158.

Jacob, F. and J. Monod. 1961. Genetic regulatory mechanisms in the synthesis of proteins. J. Mol. Biol. 3:318-356.

Jacob, F., A. Ullman, and J. Monod. 1964. Le promoteur, élement génétique nécessaire à l'expression d'un operon. C. R. Acad. Sci. (Paris) 258:3125-3128.

Jacob, F. and E. L. Wollman. 1961. Sexuality and the genetics of bacteria, pp. 191, 311; 10. Academic Press, New York.

Jacob, F. and E. L. Wollman. 1958. Les épisomes, éléments génétiques ajoutés. C. R. Acad. Sci. (Paris) 247: 154-156.

Jordon, R. C., S. E. Jacobs, and H.E.F. Davies. 1947. Studies in the dynamics of disinfection. VIII. The effect of lethal temperatures on standard cultures of Bact. Coli. I. A detailed analysis of the variations of death-rate with time. J. Hyg. (London) 45:136-143.

Kang, S. S. 1970. A mutant of Escherichia coli with temperature-sensitive streptomycin protein. Proc. Nat. Acad. Sci. U.S.A. 65:544-550.

Kauffmann, F. 1966. The bacteriology of Enterobacteriaceae. The Williams and Wilkins Co., Baltimore.

Kauffmann, F. 1961. The Bakteriologie der Salmonella-Species. Einar Munksgaard, Copenhagen.

Kauffmann, F. 1954. Enterobacteriaceae, 2nd ed. Einar Munksgaard, Copenhagen.

Kauffmann, F. 1951. Enterobacteriaceae, 1st ed. Einar Munksgaard, Copenhagen.

Kauffmann, F. 1941. Die Bakteriologie der Salmonella-Gruppe. Einar Munksgaard, Copenhagen.

Kauffmann, F. 1931. Der heutige Stand der Paratyphus-forschung. Zentralb. ges. Hyg. 25:273-311.

Kauffmann, F. 1930a. Ein kombiniertes Anreicherungsverfahren für Typhus - und Paratypnusbacillen. Zentralb. Bakteriol. Parasitenk. Abt. I. Orig. 119: 148-152.

Kauffmann, F. 1930b. Neue serologische Typen der Paratyphus-Gruppe. Z. Hyg. 111:221-232.

Kauffmann, F. 1930c. Der Antigen-Aufbau der Typhus-Paratyphus-Gruppe. Z. Hyg. 111:233-246.

Kauffmann, F. 1930d. Die kulturelle Differentialdiagnose der Typhus-Paratyphus-Gruppe. Z. Hyg. 111:247-255.

Kauffmann, F. 1929a. Zur Frage der Typ-Unwandlung von Paratyphus C in Gärtner-Bacillen. Z. Hyg. 110: 556-566.

Kauffmann, F. 1929b. Paratyphöse Gastroenteritis durch Bacillen der Paratyphus C (Suipestifer-Voldagsen)-Gruppe und durch Schottmüller-Bacillen. Z. Hyg. 109:427-433.

Kauffmann, F. 1929c. Nachtrag zu der Arbeit "Paratyphöse Gastroenteritis durch Bacillen der Paratyphus C (Suipestifer - Voldagsen)-Gruppe und durch Schottmüller-Bacillen". Z. Hyg. 109:491-492.

Kauffmann, F., F. L. Krüger, O. Lüderitz, and O. Westphal. 1961. Zur Immunchemie der O-Antigene von Enterobacteriaceae. VI. Vergleich der Zuckerbausteine von Polysacchariden aus Salmonella-S-und-R-Formen. Zentralb. Bakteriol. Parasitenk. Abt. I. Orig. 182: 57-66.

Kauffmann, F., O. Lüderitz, H. Stierlin, and O. Westphal. 1960. Zur Chemie der O-Antigene von Enterobacteriaceae. I. Analyse der Zuckerbausteine von Salmonella-O-Antigenen. Zentralb. Bakteriol. Parasitenk. Abt. I. Orig. 178:442-458.

Kauffmann, F. and C. Mitsui. 1930. Zwei neue Paratyphustypen mit bisher unbekanntem Phasenwechsel. Z. Hyg. 111: 740-748.

Kelterborn, E. 1967. Salmonella species. First isolations, names, and occurrence. Dr. W. Junk, The Hague, The Netherlands.

Koffler, H. 1957. Protoplasmic differences between mesophiles and thermophiles. Bacteriol. Rev. 21:227-240.

Kunz, L. J. and T. G. Ouchterlony. 1955. Salmonellosis originating in a hospital. A newly recognized source of infection. New Engl. J. Med. 253:761-763.

Lederberg, J., E. M. Lederberg, N. D. Zinder, and E. R. Lively. 1951. Recombination analysis of bacterial heredity. Cold Spring Harbor Symp. Quant. Biol. 16:413-441.

Lederberg, J. and E. L. Tatum. 1946a. Novel genotypes in mixed cultures of biochemical mutants of bacteria. Cold Spring Harbor Symp. Quant. Biol. 11:113-114.

Lederberg, J. and E. L. Tatum. 1946b. Gene recombination in E. coli. Nature (London) 158:558.

Lennox, E. S. 1955. Transduction of linked genetic characters of the host by bacteriophage Pl. Virology 1: 190-206.

Lieb, M. 1966. Studies on heat inducible λ mutants. II. Production of C_I product by superinfecting λ+ in heat inducible $^{-}$ lysogens. Virology 29:367-376.

Lilleengen, K. 1948. Typing Salmonella typhimurium by means of bacteriophage. Acta Pathol. Microbiol. Scand., Suppl. 77.

Lüderitz, O., A. M. Staub, and O. Westphal. 1966. Immunochemistry of O and R antigens of Salmonella and related Enterobacteriaceae. Bacteriol. Rev. 1: 192-255.

Luria, S. E. 1947. Recent advances in bacterial genetics. Bacteriol. Rev. 11:1-40.

Luria, S. E. and J. E. Darnell. 1967. General virology, 2nd ed., pp. 25, 271-281. John Wiley & Sons, Inc., New York.

Luria, S. E. and M. Delbrück. 1943. Mutations of bacteria from virus sensitivity to virus resistance. Genetics 20:491-511.

Mäkelä, P. H. 1966. Genetics determination of the O antigens of Salmonella groups B (4,5,12) and C_1(6,7). J. Bacteriol. 91:1115-1125.

Mäkelä, P. H. 1965. Inheritance of the O antigens of Salmonella groups B and D. J. Gen. Microbiol. 41:57-65.

Mäkelä, P. H. 1964. Genetic homologies between flagellar antigens of Escherichia coli and Salmonella abony. J. Gen. Microbiol. 35:503-510.

Mäkelä, P. H. and O. Mäkelä. 1966. Salmonella antigen 12_2: Genetics of form variation. Ann. Med. Exper. Biol. Fenn. 44:310-317.

Martin, W. J. and W. H. Ewing. 1969. Prevalence of serotypes of Salmonella. Appl. Microbiol. 17:111-117.

McDonald, W. C. and T. S. Matney. 1963. Genetic transfer of the ability to grow at 55°C in Bacillus subtilis. J. Bacteriol. 85:218-220.

Mefferd, R. B., Jr. and L. L. Campbell, Jr. 1952. Studies on a thermophilic variant of Bacillus globigii. Texas Rep. Biol. Med. 10:419-420.

MH Bulletin. 1961. Salmonella organisms in animal feeding stuffs. Pub. Hlth. Lab. Serv., Monthly Bull. Minist. Hlth. (London) 20:73-85.

Milone, N. A. and J. A. Watson. 1970. Thermal inactivation of Salmonella senftenberg 775W in poultry meat. Health Lab. Sci. 7:199-225.

Miyake, T. 1962. Exchange of genetic material between Salmonella typhimurium and Escherichia coli K-12. Genetics 47:1043-1052.

Miyake, T. and M. Demerec. 1959. Salmonella-Escherichia hybrids. Nature (London) 183:1586.

Moran, A. B., C. D. Van Houweling, and E. M. Ellis. 1965. The results of typing Salmonella from animal sources in the United States. In NCDC, 1965. Ibid., pp. 33-40.

MRC Report. 1947. The bacteriology of spray-dried eggs with particular reference to food poisoning. Med. Res. Counc. Gt. Brit., Spec. Rep. Ser. No. 260. H.M.S.O., London.

Naide, Y., H. Nikaido, P. H. Mäkelä, R. G. Wilson, and B.A.D. Stocker. 1965. Semirough strains of Salmonella. Proc. Nat. Acad. Sci. U.S.A. 53:147-153.

Newell, K. W., R. McClaren, C. R. Murdoch, W. N. MacDonald, and H. L. Hutchinson. 1959. Salmonellosis in Northern Ireland with special reference to pigs and Salmonella-contaminated pig meal. J. Hyg. (London) 57:92-105.

NCDC. 1965. Proc. Natl. Conf. on Salmonellosis (Atlanta, 1964). Natl. Commun. Dis. Center Publ., Atlanta, Ga.

Ng, H., H. G. Bayne, and J. A. Garibaldi. 1969. Heat resistance of Salmonella: The uniqueness of Salmonella senftenberg 775W. Appl. Microbiol. 17:78-82.

Ng, H. 1966. Heat sensitivity of 300 Salmonella isolates In ARS Report, 1966. The destruction of salmonellae. Ibid., pp. 39-41.

Nomura, M., S. Mizushima, M. Ozaki, P. Traub, and C. V. Lowry. 1969. Structure and function of ribosomes and their molecular components. Cold Spring Harbor Symp. Quant. Biol. 34:49-61.

Northrup, J. and R. A. Slepecky. 1967. Sporulation mutations induced by heat in Bacillus subtilis. Science 155: 838-830.

NRC Report. 1969. An evaluation of the Salmonella problem. NAS-NRC Publ. No. 1683, Natl. Acad. Sci. Natl. Res. Counc., Washington, D.C.

Olitsky, I., H. Rosenthal, and J. R. Copeland. 1956. Family infection with multiple Salmonella types including two H_2S-negative variants. J. Bacteriol. 72:569-570.

Ørskov, F., I. Ørskov, and F. Kauffmann. 1961. The fertility of Salmonella strains determined in mating experiments with Escherichia strains. Acta Pathol. Microbiol. Scand. 51:291-296.

Osborne, W. W., R. P. Straka, and H. Lineweaver. 1954. Heat resistance of strains of Salmonella in liquid whole egg, egg yolk, and egg white. Food Res. 19:451-463.

Ozaki, M., S. Mizushima, and M. Nomura. 1969. Identification and functional characterization of the protein controlled by the streptomycin-resistant locus in E. coli. Nature (London) 222:333-339.

Ozaki, H., B.A.D. Stocker, and S. M. Smith. Transmission of colicinogeny between strains of Salmonella typhimurium grown together. J. Gen. Microbiol. 28:671-687.

Pace, B. and L. L. Campbell. 1967. Correlation of maximal growth temperature and ribosome heat stability. Proc. Natl. Acad. Sci. U.S.A. 51:1110-1116.

Pomeroy, B. S. and M. Grady. 1960. The isolation of Salmonella organisms from feedstuffs. Proc. 3rd Natl. Symp. on Nitrofurans in Agric., University of Kentucky, p. 158.

Prost. E. and H. Riemann. 1967. Foodborne salmonellosis. Annu. Rev. Microbiol. 21:495-528.

Rossett, R. and L. Gorini. 1969. A ribosomal ambiguity mutation. J. Mol. Biol. 39:95-112.

Sanderson, K. E. 1967. Revised linkage map of Salmonella typhimurium. Bacteriol. Rev. 31:354-372.

Sanderson, K. E. and M. Demerec. 1965. The linkage map of Salmonella typhimurium. Genetics 51:897-913.

Sanderson, E. S. and M. J. Lewis. 1965. Drug resistance and its transfer in Salmonella typhimurium. Nature (London) 206:579-583.

Sarvas, M. and P. H. Mäkelä. 1965. The production, by recombination, of Salmonella forms with both T_1 and O specificities. Acta Pathol. Microbiol. Scand. 65: 654-656.

Saunders, G. F. and L. L. Campbell. 1966. Ribonucleic acid and ribosomes of Bacillus stearothermophilus. J. Bacteriol. 91:332-339.

Savage, W. G. 1932. Some problems of Salmonella food poisoning; tenth William Thompson Sedgwick memorial lecture. J. Prev. Med. 6:425-451.

Schmidt, C. F. 1957. Thermal resistance of microorganisms. In G. F. Reddish (ed.), Antiseptics, disinfectants, fungicides and sterilization, 2nd ed., pp. 831-844. Lea and Febiger, Philadelphia.

Seligmann, E., I. Saphra, and M. Wassermann. 1943. Salmonella infections in man. An analysis of 1,000 cases bacteriologically identified by the New York Salmonella Center. Amer. J. Hyg. 38:226-249.

Smith, D. H. 1966. Salmonella with transferable drug resistance. New Engl. J. Med. 275:625-630.

Smith, H. W. and A. Buxton. 1951a. Isolation of salmonellae from faeces of domestic animals. Brit. Med. J. 1: 1478-1483.

Smith, H. W. and A. Buxton. 1951b. An outbreak of Salmonella schwartzengrund infection in poultry. J. Pathol. Bacteriol. 63:459-463.

Smith, D. T., N. F. Conant, and H. P. Willet. 1968. Zinsser microbiology, 14th ed., pp. 80-82. Appleton-Century-Crofts, New York.

Smith, H. O. and M. Levine. 1965. Gene order in prophage P22. Virology 27:229-231.

Smith, S. M. and B.A.D. Stocker. 1962. Colicinogeny and recombination. Brit. Med. Bull. 18:46-51.

Sogin, S. J. and Z. J. Ordal. 1967. Regeneration of ribosomes and ribosomal ribonucleic acid during repair of thermal injury to Staphylococcus aureus. J. Bacteriol. 94:1082-1087.

Solowey, M., V. H. McFarlane, E. H. Spaulding, and C. Chimerda. 1947. Microbiology of spray-dried whole egg. II. Incidence and types of Salmonella. Amer. J. Pub. Health 37:971-982.

Staub, A. M., R. Girard. 1965. X. Analyse des facteurs 1 des groupes B, E_1 et G: leur rapport avec les facteurs I_{12}, 19 et 37. Bull. Soc. Chim. Biol. 47:1245-1268.

Stent, G. S. 1971. Molecular genetics, 1st ed., pp. 84-109; 108; 337, 373-374; 171-172; 378; 595. W. H. Freeman and Co., San Francisco.

Stern, W. 1916. Studien zur differenzierung der Bakterien der Coli Typhus Gruppe mittles gefärbter flüssiger Nähroböden. Beitrage zur Biologie der Bakteriengruppe Paratyphus B-Enteritidis. Zentralb. Bakteriol. Parasitenk. Abt. I. Orig. 78:481-492.

Stumbo, C. R. 1965. Thermobacteriology in food processing, pp. 92-102. Academic Press, Inc., New York.

Subcommittee on Salmonella. 1940. Report of the Subcommittee of the Nomenclature Committee of the International Association of Microbiologists on the Genus Salmonella. Proc. 3rd Internatl. Cong. Microbiol. (New York), pp. 832-840.

Subcommittee on Salmonella. 1934. The genus Salmonella Lignierès, 1900. J. Hyg. (London) 3:333-350.

Sugihara, T. F., K. Ijichi, and L. Kline. 1966. Heat pasteurization of liquid whole egg. Food Technol. 20:1076-1083.

Tai, P. C., D. P. Kessler, and J. Ingraham. 1969. Cold-sensitive mutations in Salmonella typhimurium which affects ribosome synthesis. J. Bacteriol. 97: 1298-1304.

Taylor, J. 1965. Salmonellosis in the United Kingdom. In NCDC, 1965. Ibid., pp. 18-26.

Taylor, J. 1962. Salmonella and salmonellosis. Food poisoning. Royal Soc. Health, London, 19:178.

Taylor, J., M. M. Lee, P. R. Edwards, and C. H. Ramsey. 1960. A new type of flagellar variation associated with new antigens in the Salmonella group. J. Gen. Microbiol. 23:583-588.

Thatcher, F. S. 1965. Large population at risk. In NCDC, 1965. Ibid., pp. 56-62.

Turri, M. et al. 1964. In M. C. Allwood and A. D. Russell, 1970. Ibid., p. 101.

Uchida, T., P. W. Robbins, and S. E. Luria. 1963. Analysis of the serologic determinant groups of the Salmonella E groups O antigens. Biochemistry 2:663-668.

Uetake, H., S. E. Luria, and S. W. Burrows. 1958. Conversion of somatic antigens in Salmonella by phage infection leading to lysis or lysogeny. Virology 5:68-91

Uetake, H., T. Nakagawa. 1955. The relationship of bacteriophage to antigenic changes in group E salmonellas. J. Bacteriol. 69:571-579.

van Oye, E. (ed.). 1964. The world problem of Salmonellosis. Dr. W. Junk, The Hague, The Netherlands.

Varela, G. and J. Olarte. 1952. Classification and distribution of 1,075 cultures of Salmonella isolated in the city of Mexico. J. Lab. Clin. Med. 40:73-77.

Varela, G. and J. Zozaya. 1942. Salmonella in the glands of pigs. Bull. Hyg. (London) 17:721.

Warren, S. H. and W. M. Scott. 1930. A new serological type of Salmonella. J. Hyg. (London) 29:415-417.

Watanabe, T. 1963. Infective heredity of multiple drug resistance in bacteria. Bacteriol. Rev. 27:87-115.

Watanabe, S., S. Minamimoto, and N. Muruse. 1952. Studies on salmonellosis in fowls. I. The isolation of S. senftenberg from died and under-developed chick-embryos. Bull. Natl. Inst. Anim. Hlth. Tokyo 24:2-5.

Watson, J. D. 1965. Molecular biology of the gene, 1st ed., pp. 168-177; 324-325; 395-404. W. A. Benjamin, Inc., New York.

Wetzler, T. F., N. R. Freeman, M.L.V. French, L. A. Renkowski, W. C. Eveland, and O. J. Carver. 1968. Biological characterization of Listeria monocytogenes. Health Lab. Sci. 5:46-62.

Wilson, G. S. and A. A. Miles. 1964. Topley and Wilson's principles of bacteriology and immunity, 5th ed., pp. 359-387; 364; 373-376, 881; 1915. The Williams and Wilkins Co., Baltimore.

Winter, A. R., G. F. Steward, V. H. McFarlane, and M. Solowey. 1946. Pasteruization of liquid egg products. III. Destruction of salmonellae in liquid whole egg. Amer. J. Pub. Health 36:451-460.

Yurack, J. A. and E.W.R. Best. 1965. Salmonellosis in Canada. In NCDC, 1965. Ibid., pp. 27-32.

Zamenhof, S. 1960. Effects of heating dry bacteria and spores on their phenotype and genotype. Proc. Nat. Acad. Sci. U.S.A. 46:101-105.

Zamenhof, S. and S. Greer. 1958. Heat as an agent producing high frequency of mutations and unstable genes in Escherichia coli. Nature (London) 182:611-613.

Zinder, N. D. 1960. Hybrids of Escherichia and Salmonella. Science 131:813-815.

Zinder, N. D. and J. Lederberg. 1952. Genetic exchange in Salmonella. J. Bacteriol. 64:679-699.