THE COMBINED EFFECT OF WATER ACTIVITY, TEMPERATURE
AND pH ON THE GROWTH OF THREE STRAINS
OF YERSINIA ENTEROCOLITICA

by

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ABSTRACT

The combined effect of water activity ($a_w$), pH and temperature on the growth of *Yersinia enterocolitica* serotypes 0:8, 0:17 and 0:22 was studied. Media were adjusted to various $a_w$ levels with sucrose, salt or glycerol. Upon establishment of the lowest test level permitting growth at 4C or 25C, the further effect of pH was observed. The pH was adjusted to 7.0, 6.0 and 5.0. Glycerol was least inhibitory; NaCl was most inhibitory. Generally, incubation at 25C permitted growth at lower $a_w$/pH combinations as compared to 4C. However, survivors were higher in number and more frequent among different $a_w$/pH combinations when incubated at 4C.
INTRODUCTION

The awareness of *Yersinia enterocolitica* as a human pathogen has increased in the last ten years. Extensive research has been conducted in several European countries on strains isolated from human, animal and environmental sources. The most prevalent sources of human infection have yet to be determined. In the past, many of the strains isolated from animals and environmental sources were of bioserotypes seldom involved with human infections. Recently, bioserotypes associated with human infections have been isolated from animal, environmental and food sources. These isolates have established food as a potential source of human infection.

The potential became a reality with the occurrence of food-borne illness outbreaks implicating *Y. enterocolitica*. Raw milk was implicated as the source of two large outbreaks of *Y. enterocolitica* infections involving 137 children and one adult in Quebec, Canada. Another outbreak, involving at least 30 children, occurred in Holland Patent, New York. Chocolate milk was determined to be the common vector of transmission in this incident.

In spite of this implication of food-borne illness potential, very little research has been done pertaining to *Y. enterocolitica* and its behavior in food systems.
Controlling the organism in foods by way of processing and preservation parameters has been minimally researched. The purpose of this study was to determine the combined effect of water activity, pH and temperature on the growth and survival of \( Y. \) enterocolitica.
LITERATURE REVIEW

History of Yersinia enterocolitica

Y. enterocolitica is a well documented organism in European countries. Y. enterocolitica has only recently attracted attention from food and industrial microbiologists and clinicians in the United States. Although the organism is only now creating interest, Schleifstein and Coleman (1943) isolated, described and biochemically characterized five clinical isolates of Y. enterocolitica in the United States in 1939.

Since the late sixties, a sharp increase in the incidence of Y. enterocolitica, especially in European countries (Mollarett 1972) has occurred. In a five year survey of human infections in Hungary, Szita and Svidro (1976) showed that 1355 strains were isolated from 1096 people. Twenty-three percent were asymptomatic excreters. There has been an increase in other European countries in the variety of chemical forms recorded, in the number of countries involved, and in the number of animal species infected (Mollarett 1972).

Renewed interest in the United States followed the isolation of Y. enterocolitica by Sonnenwirth (1968) from a case of meningitis. In the last decade, Bottone (1977) reported that the incidence of Y. enterocolitica isolations from human and nonhuman sources as gradually increased.
The isolation and similar increase of infections of *Y. enterocolitica* in Japan and Canada has paralleled the recent awareness of this organism in the United States. By 1978, isolations had been made in Canada from humans (Capriolli, Drapeau and Kasatiya 1978), animals (Toma and Deidrick 1975), foods (Schiemann and Toma 1978) and water (Capriolli et al. 1978). In Japan, Zen-Yoji and Maruyama (1972) described the first isolations and identifications of *Y. enterocolitica* from human sources. In 1972, two outbreaks of human yersiniosis were documented by Asakwa et al. (1973). In the two separate outbreaks, approximately 1100 children displayed signs of the disease. Although no vector of transmission was established, food and/or water were implicated. Since these Japanese outbreaks occurred, *Y. enterocolitica* has been isolated from swine (Tsubokura, Otsuki and Itagaki 1973) and cattle in Japan (Inoue and Kurose 1975, Zen-Yoji et al. 1974).

There is a worldwide increase of this organism. The more research that is performed and data gathered on *Y. enterocolitica*, the more obvious it becomes that this organism is an important human pathogen (Lee 1977).

**Description of the Bacterium Yersinia enterocolitica**

The genus was proposed by von Loghem in 1944 to separate from the genus *Pasteurella* those bacteria that differed from the septicemic group *Pasteurella sensu stricto*. The genus name was taken from the French
bacteriologist A. J. E. Yersin who first isolated the casual organism of plague in 1894. In 1954, Thal proposed that the genus *Yersinia* should be included in the family *Enterobacteriaceae*. Numerical methods seem to justify the division of *Pasteurella* (Smith and Thal 1965) and the relation of *Yersinia* to the *Enterobacteriaceae*.

The bacterium was classified as *Pasteurella* X. Frederiksen (1964) proposed the new species name *enterocolitica*, pertaining to the intestine and colon. *Y. enterocolitica* is now considered a member of the family *Enterobacteriaceae* and has been classified as such in the *Bergey's Manual of Determinative Bacteriology* (1974):

*Y. enterocolitica* is a facultative, anaerobic, Gram-negative rod. Coccoid forms are predominant in young cultures at 22-25°C. There is a marked tendency to pleomorphism in older cultures (Nilehn 1969a), especially in those grown at 37°C or on various selective media. On first isolation a smooth colony forms, 0.5-2.0 mm in diameter. In subculture dissociation occurs and R forms are frequent.

According to Knapp and Thal (1963), the optimum temperature for growth of the organism is 30-37°C, while Mollaret, Chevalier and Deplanche, (1964) did not find any appreciable difference in growth at 18°C, 28°C, and 37°C. In preliminary studies on one recently isolated strain of human origin grown in nutrient broth at different temperatures, the shortest average generation time was found on
incubation at 34C (Nilehn 1968). Growth rate declined on incubation at 39C, 30C, 25C and 4C, respectively. No significant difference in growth rate was observed (Chester and Stotzky 1975) when grown at 22C and 37C. After various incubation periods, the colony counts and growth curves demonstrated that no significant variation occurred in the rate of growth of three strains of \textit{Y. enterocolitica} at 22C and 37C other than a one hour lag in reaching logarithmic. Good growth has been reported at 4C (Mollaret et al. 1964, Nilehn 1969a, Hughes 1978, Lee 1977, Hanna et al. 1976, Greenwood et al. 1975). This ability to grow at 4C poses a significant potential public health problem. Insufficiently processed and/or stored foods contaminated with this organism could present a hazard.

There is a tremendous variation of biochemical, serological, physiological and genetic characteristics among strains of \textit{Y. enterocolitica}. Biochemical tests have been used to establish different groups or biotypes (Nilehn 1969b, Knapp and Thal 1973) for \textit{Y. enterocolitica}. It has also been suggested (Knapp and Mollaret 1970, Brenner et al. 1976) that the biotypes be divided into three or four new species.

Although no such division has taken place, \textit{Y. enterocolitica} has been unofficially (Chester and Stotzky 1975, Bottone et al. 1974, Weaver and Jordan, 1973) divided into typical and atypical categories in the United States. This division is based on the biochemical ability or
inability to ferment rhamnose. The studies revealed a definite temperature dependence (positive at 25°C; negative at 37°C) of specific biochemical tests for the rhamnose positive isolates (Y. e. \( \text{rh}^+ \)). The Y. e. \( \text{rh}^+ \) clinical isolates appeared to be identical, biochemically and culturally with Y. enterocolitica 0:17, isolated in Denmark (Lassen 1972) from fecally contaminated drinking water. Hence, the environmental strains were designated as atypical Y. enterocolitica. Other investigators (Hanna et al. 1976, Bottone 1977, Restaino et al. 1979) have pointed out this temperature dependent characteristic. With a recent, rapid increase of atypical isolates from clinical (Bottone et al. 1974, Kapperud 1977, Lassen 1972) and food (Hanna et al. 1976, Hughes 1978, Schiemann and Toma 1978) sources, the public health significance of the atypical strains has increased.

Many investigators (Nilehn 1969a, Bottone 1977, Hanna et al. 1976) have cited data that points to temperature dependence of biochemical tests for both typical and atypical strains for Y. enterocolitica. The biochemical reactions most often cited as temperature dependent are beta-galactosidase, Voges-Proskauer, motility, citrate utilization and rhamnose fermentation.

The serology of the species is being developed. At present both O and H antigens have been demonstrated. Winbald (1968) originally identified eight O antigens. Some strains have another O antigen related to Brucella abortus.
The H and O antigens are common to strains from humans and animals (Knapp and Thal 1963) and are not related to those of Yersinia pseudotuberculosis.

Several studies (Nilehn 1967; Nilehn 1969a; Wauters 1973; Winbald; Nilehn and Sternby 1966) have shown the importance of the serology of this bacterium. These studies have all established possible links between human infection and the source of that infection. Thirty-four O-antigen serotypes (Wauters 1973) of Y. enterocolitica have been established.

To date, the only published work involving physiological responses to the environment (foods) has been done by Hanna et al. of Texas A&M University. Some areas studied which showed strain variation were the effects of growth temperature, heating and freezing. The parameters will be discussed individually.

(i) Temperature. With two atypical strains and one typical strain, an average of a two log increase occurred on raw beef held over a ten day period at 0-1C. Counts were similar. However, when the incubation temperature was increased, one of the atypical strains outgrew the other two strains. When inoculated raw or cooked beef and pork were stored at 7C and 25C, increases of 4-5 logs occurred for all strains but one atypical strain. The increases were somewhat greater on cooked than raw product. The authors (Hanna et al. 1977c) gave two possible explanations for this, differences
in the physicochemical characteristics of the product and
differences in the level and type of microbial flora that
developed. Staphylococci and micrococci predominated
on cooked product, whereas, pseudomonas was more common on
raw product.

(ii) Heating. With a final internal temperature
of 55-62C in beef roasts, no survivors were present in 100g
of beef. In one case, a final internal temperature of 51C
yielded survivors. The surviving strain was atypical
(Hanna et al. 1977a). The one typical strain tested did
not survive. These results were consistent with data
obtained from heated skim milk (Hanna et al. 1977b). Data
here indicated considerable variation in the heat resistance
of five strains. All five strains survived ten minutes at
50C. The two typical strains were somewhat more heat
resistant at 50C, 55C, and 60C than the three atypical
strains. The typical strains survived heating at 55C for
ten minutes, whereas none of the atypical strains survived.

(iii) Freezing. Extensive reductions occurred
during frozen storage of beef samples (Hanna et al. 1977a)
for all strains tested. However, the typical strain was
less affected than the atypical strain.

Genetic studies on _Y. enterocolitica_ have been some-
what limited. Brenner et al. (1976) characterized strains
of _Y. enterocolitica_ and _Y. pseudotuberculosis_ by deoxy-
ribonucleic acid (DNA) hybridization, and the extent of DNA
relatedness between the strains was assessed. *Y. pseudotuberculosis* was only 40 to 60% related to *Y. enterocolitica*. *Y. enterocolitica* strains formed three DNA relatedness groups, and the data strongly suggested a fourth group. Each group could be defined biochemically. One group corresponded to typical *Y. enterocolitica*; the second was rhamnose positive (atypical); the third was rhamnose and raffinose positive. The suggested fourth was sucrose negative. The study concluded that, although all of the DNA relatedness groups should remain in the genus Yersinia, only the first group is *Y. enterocolitica*. It was also shown that all the yersiniae tested were distantly, but significantly, related to other members of Enterobacteriaceae.

Cornelis (1975) and Cornelis and Abraham (1975) described beta-lactamases synthesized by *Y. enterocolitica*. These lactamases were isolated from six strains and played a role in the resistance of the species to beta-lactam antibiotics.

The production of a heat stable exotoxin (Pai and Mors 1978) has been established. Typical strains of *Y. enterocolitica* were shown to produce a heat stable exotoxin similar to that of the toxin produced by enteropathogenic *Escherichia coli*. These findings increase the probability of these strains becoming an important human food-borne pathogen.
Epidemiology of *Yersinia enterocolitica*

The organism is ubiquitous among warm blooded animals having been isolated from cattle, swine, dogs, sheep, rabbits, horses, guinea-pigs and deer (Ahvonen, Thal and Vasenious 1973, Inoue and Kurose 1975, Langford 1972, Toma and Deidrick 1975, Tsuborkura et al. 1973, Zen-Yoji et al. 1974). The organism has been isolated from mesenteric lymph nodes, feces and intestinal contents of diseased and healthy animals and man. Recently, this organism has been found in various food sources; milk (Hughes 1978, Morbidity and Mortality Weekly Report [MMWR] 1977) beef (Inoue and Kurose 1975) vacuum-packaged beef and lamb (Hanna et al. 1976) and water (Lassen 1972, Harvey et al. 1976).

There are presently over 6,000 European isolates of *Y. enterocolitica* at the Institut Pasteur (Bottone 1977). Incidence of *Y. enterocolitica* in the United States and Canada is not as prevalent as in Europe. Documentation of isolates from humans and nonhuman sources is still a case-to-case basis in Canada and the United States. Although 84 cases of yersiniosis have been reported since 1968, the incidence of this disease in the United States is far below that which has been observed in other areas of the world. At present, the organism has been isolated in Europe, North America, Africa, Asia and Australia.

An epidemiologically important aspect of the bacterium pertains to its geographical distribution of specific somatic
antigen serotypes. The predominant serotypes isolated from humans are 0:8, found in the United States, and 0:3 and 0:9 found in Japanese, European and Canadian cultures. *Y. enterocolitica* serotype 0:3 is the predominant strain isolated from European (Esseueld and Goudzwaard, 1973) and Canadian (Toma and Deidrick 1975, Toma 1973) swine. The Japanese (Tsubokura et al. 1973, Zen-Yoji et al. 1974, Zen-Yoji and Maruyama 1972) have isolated serotype 0:3 and 0:9 from swine, cow's intestine and beef meat. The predominant Canadian serotype, 0:3, differs from the European and Japanese isolates in that it is phage type 9b (Bottone 1977).

Bissett (1976) has the distinction of isolating the only two 0:3 serotypes from humans in the United States. The infrequency of serotype 0:8 isolates other than the United States also holds true. Capriollì et al. (1978) are the exception with 0:8 isolates from humans in Canada. Presently, no study has established the isolation of a single 0:8 strain from humans in Europe or Japan.

Another unique characteristic of *Y. enterocolitica* in the United States pertains to the isolation of the previously discussed atypical strains from humans. These strains are usually classified (Bottone et al. 1974) as serotype 0:17. These unusual strains, which produce various clinical entities such as urinary tract infections, localized skin abscesses and wound infections, have not been isolated.
humans in Canada, Japan or Europe. These strains have, however, been isolated from nonhuman sources in the United States (Hanna et al. 1976).

Epidemiology relative to transmission of yersiniosis to man is not clearly understood. It has been suggested that the major mode of transmission occurs through one of the following: foods contaminated by feces or urine (Toma and Lafleur 1974), direct contact with an infected animal (Rabson, Hullet and Koornhof 1975) or person-to-person transmission (Gutman et al. 1973; Szita, Kali and Redey 1973). Direct contact with an infected animal or person does not seem to be the primary mode of transmission of the disease to man. In the recorded incidents, the number of cases was small. Transfer of yersiniosis from person-to-person not in close proximity and constant contact (Asakwa et al. 1973; Szita et al. 1973) is quite low. These two vehicles of transmission usually involve sporadic cases and limited family outbreaks.

The mode of transmission that could have the most serious ramifications is contaminated foods. Serotypes identical to those recovered from human hosts have been isolated from animals, particularly swine (Toma and Deidrick 1975, Zen-Yoji et al. 1974, Esseueld and Goudyzwaard 1973). Investigators in Europe, Japan and Canada have unequivocally demonstrated that swine are the major reservoir for serotype 0:3 strains ultimately isolated in
humans (Toma and Deidrick 1975, Bottone et al. 1974).

Szita et al. (1973) demonstrated a history of pork ingestion or direct contact with swine in patients with yersiniosis.

Table 1 illustrates evidence linking various sources as the transmission vectors of \( Y. \) enterocolitica to humans. In Canada (Capriolli et al. 1978, Schieman and Toma 1978), human serotypes have been isolated from pancakes, cheese, raw milk, ham, beef, and water.

The atypical strains have also been isolated from nonmesenteric clinical materials (Bottone et al. 1974). These atypical strains were isolated from water. Recently, they have been isolated from meats and patients with limited enterocolitis or nonmesenteric lymphadenitis. According to Bottone et al. (1974), in contrast to typical \( Y. \) enterocolitica, which has become well adapted in animal and human hosts, it appears that environmental strains may be in the evolutionary process of becoming adapted to humans.

The bacterium can produce a patterned clinical symptomatology in human beings (Asakwa et al. 1973; Gutman et al. 1973; MMWR 1977; Nilehn 1969b; Szita et al. 1973). An acute gastroenteritis or enterocolitis is the initial phase of the disease, usually followed by an acute response of the right iliac fossa eliciting one of the following: pseudoappendicitis, mesenteric lymphadenitis of terminal ileitis. Enterocolitis is the most frequently encountered manifestation of yersiniosis. This is characterized by
Table 1. Evidence Linking Food Sources as the Transmission Vector of *Yersinia enterocolitica*

<table>
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<th>Serotype isolated from human sources</th>
<th>Environmental origin of human serotype</th>
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<tr>
<td>Serotype 0:3</td>
<td>Intestinal origin and feces from swine (Canada, Japan and Netherlands)</td>
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<tr>
<td>Serotype 0:5</td>
<td>Vacuum-packaged beef and lamb (United States)</td>
</tr>
<tr>
<td>Serotype 0:8</td>
<td>Intestinal contents from cows (Japan)</td>
</tr>
<tr>
<td>Serotype 0:9</td>
<td>Beef, ham, sausage meat (Japan and Canada)</td>
</tr>
<tr>
<td>Serotype 0:17 (atypical)</td>
<td>Milk (United States and Canada)</td>
</tr>
<tr>
<td></td>
<td>Water (United States, Canada and Norway)</td>
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diarrhea lasting from two days to several weeks. Vomiting may be present and the body temperature is elevated (greater than 39°C).

**Water Activity: Theory and Practice**

Water activity ($a_w$) is defined as the ratio of water vapor pressure above the food to the vapor pressure of pure water with an assigned value of one. An equilibrium relative humidity value of 50% corresponds to a $a_w$ value expressed as 0.50. From the viewpoint of the food microbiologist, $a_w$ indicates the amount of water in a food system which is available for micro-organisms. This is not the total water content of the food because a proportion of it may be bound by water soluble salts, proteins and carbohydrates. This bound water is not available to micro-organisms. Osmotic shock, insufficient availability of required nutrients or a build-up of metabolic end-products that are toxic to the organism are thought to be some of the means by which $a_w$ limits or inhibits the growth of micro-organisms (Warmbier, Schnickels and Labuza 1976).

The $a_w$ of a food influences the multiplication (Scott 1953), metabolic activity (including toxin production), survival and resistance of micro-organisms. This is true not only for organisms that cause spoilage and food-poisoning, but also for those which are desirable for the fermentation of certain foods. Microbial spoilage, food-poisoning and fermentation take place if the $a_w$ of the substrate is
favorable for the multiplication and metabolic activity of the organisms involved (Leistner and Rodel 1976). Most organisms occurring in foods proliferate at a high $a_w$. Only a few require a low $a_w$ for growth. Thus, if the $a_w$ decreases, then fewer general of micro-organisms are able to multiply on or in a food. A decrease in the $a_w$ of a food and, thereby an extension of its storage life, can be accomplished by drying, salting, addition of sugar or freezing. Some of these processes are applied in combination. Furthermore, inhibition of micro-organisms in a food is frequently not caused solely by a decrease in $a_w$, but may be influenced by pH, temperature, preservatives or a combination thereof (Troller 1973).

In general, of the micro-organisms associated with foods, molds are more tolerant of decreased $a_w$ than yeasts, and yeasts more tolerant than bacteria. In high moisture foods ($a_w > 0.90$) bacteria are mainly responsible for spoilage, food-poisoning or fermentation. In intermediate moisture foods ($a_w 0.90-0.60$) yeasts and molds are of significance in spoilage. However, most micro-organisms are inhibited in low moisture foods ($a_w < 0.60$).

Insalata (1972) states that microbiological growth is controlled by $a_w$ using the effects of moisture equilibrium reaction, reaction kinetics, adsorption, desorption and nutritional requirements of the microbial population present. The amount of water present and the type and
concentration of osmotically active substances (salt, sucrose and glycerol) in the system are used to control $a_w$ (Haas et al. 1975). Not only the obtained $a_w$ level, but also moisture content determine microbial activity (Haas et al. 1975, Labuza, Cassil and Sinskey 1972, Plitman et al. 1973). The solutes lower availability of water by binding it (Sloan, Waletzko and Labuza 1976), making it unavailable chemically and biologically. These solutes also increase viscosity of the liquid phase, therefore, lowering reactant diffusion rates (Labuza, 1968, 1971, 1974).

As $a_w$ is reduced from its optimum, there is generally an increase in the logarithmic growth phase of the organism and a decrease in the amount of cell material synthesized resulting in reduced growth rate and the organisms subsequent death (Insalata 1972). Haas et al. (1975) and Kang et al. (1969) showed that at lower $a_w$ levels, lag phases were extended, smaller populations at the maximal growth stage were obtained and faster death rates were recorded.

Greatest tolerance to low water activity occurs at optimum incubation temperatures for the organism involved. At temperatures above or below optimum, range of $a_w$ permitting microbial growth is reduced. Insalata (1972) showed that in an ideal solution, with pressure and composition constant, $a_w$ is completely independent of temperature. In
practical application, however, these criteria are seldom met.

The pH of a food system is related to $a_w$ and, therefore, microbial control. Minimum $a_w$ permitting growth appears to increase as pH decreases (Ohye and Christian 1966, Genigeorgis et al. 1971). The source of acid is important. Nunheimer and Fabian (1940) concluded that organic acids were less effective when combined with salt than inorganic acids. These studies point out a definite reduction in growth when pH is used in combination with lowered $a_w$ levels.

Humectants can be added into a system either in a dry state or as a solution by previously dissolving the humectant in water. As a result of the method of addition of water into a system, i.e., adsorption vs. desorption, a hysteresis or order-of-mixing phenomenon may occur (Rao 1941; Taylor, Chiskey and Senti 1961; Betterleheim and Ehrlich 1963; Berlin, Anderson and Pollausch 1969; Mackenzie and Luyet 1971). Sloan et al. (1976) concluded even though the pure humectant water isotherm may exhibit a hysteresis effect, there is no significant difference in the final $a_w$ achieved as a result of the order-of-mixing in a food system. Sloan et al. (1976) also exhibited no hysteresis effect for glycerol and sucrose in the pure water system. An additional point to be considered here is the effect on the bacterial population of this order-of-mixing. It has been
demonstrated (Plitman et al. 1973) that the desorption technique (addition of humectant to system until desired $a_w$ level is reached) yields a lower death rate.

The type of solute used will vary the effects on cells observed and the amount of solute needed to achieve desired $a_w$ levels. The solute used will produce differences in growth rate and in lowest $a_w$ permitting growth (Kang et al. 1969). Glycerol, due to its use in pet foods, has been a well studied humectant. Glycerol has a much lower vapor pressure (Sloan et al. 1976) which in turn lowers $a_w$ (Karmas and Chen 1975). Glycerol does not seem to have as great a bacteriocidal effect as the polyols (Plitman et al. 1973) or other humectants. This lower bacteriocidal effect seems to be related to the negligible water binding capacity of glycerol. Karmas and Chen (1975) used differential scanning calorimetry to demonstrate this low binding. The energy of hydrogen bonding between glycerol and water is not significantly different from the energy of existing water molecules, therefore, water molecules are not appreciably bound by glycerol. Another explanation for the lowered inhibitory properties of glycerol is that it easily enters the cell and prevents an osmotic imbalance (Charlang and Horowitz 1971).

Salt and sucrose are more inhibitory to bacteria when compared to glycerol. Smaller amounts of each solute are needed to reduce $a_w$ to inhibitory levels. Charlang and Horowitz (1971) suggested that the difference is due to the
electrolytic properties of these solutes. The ionic bonding between these solutes and water may be responsible for increasing water binding and lowering $a_w$. For a sodium chloride solution (Karmas and Chen 1975) the percentage of bound water was strongly correlated to a lowering of $a_w$. Sodium chloride has a small molecular weight and, in an aqueous solution, it depresses $a_w$ uniformly throughout the solution. The ionic bonding between the sodium and chloride ions and water were suggested as the principle reason for significant lowering of $a_w$ by salt.

To date, no published data involving minimal water activities for growth of *Yersinia enterocolitica* are available. Much data has been gathered on Gram-negative organisms which are also in the family Enterobacteriaceae. The remainder of this review will be used to discuss some $a_w$ limits established with similar organisms. This is only intended to give an idea of limits which may be expected for *Y. enterocolitica*. Christian and Scott (1953) grew 16 strains of *Salmonella* in casamino acids-yeast extract-casitone medium adjusted to various $a_w$ levels by a mixture of NaCl, KCl and Na$_2$SO$_4$. Growth occurred at $a_w$ levels between 0.999 and 0.945 with an average optimum of 0.995. Reductions of $a_w$ below 0.99 produced an increase in lag period as well as in total cell yield, regardless of whether growth was aerobic or anaerobic.

Using sucrose, glucose, NaCl or KCl to control $a_w$, Christian (1955b) found that *Salmonella oranienburg* grew at
\( a_w \) 0.97, regardless of which solute was used to adjust the \( a_w \). Only glycerol allowed growth at 0.96; it did not allow growth at 0.95.

In studies on the influence of nutritional factors on the limiting water for \( S. \) oranienburg, Christian (1955a) noted that the lower \( a_w \) limit for growth in three complex media was between 0.94 and 0.95 but was slightly higher (0.96 to 0.97) in a defined medium. Upon addition of proline and methionine to the defined medium, growth was obtained at 0.96. Similarly, addition of eight water-soluble vitamins plus the above amino acids further extended the minimal \( a_w \) range to 0.95.

Sakazaki, Iwanami and Fukumi (1963) has reported that growth of \textit{Vibrio parahaemolyticus} is easily obtained in culture media to which 1 to 3% NaCl has been added, with optimal growth occurring in the 2-4% range. Maximal salt concentration at which growth will occur is in the 8-9% range.

According to LeClaire, Zen-Yoji and Sakai (1970), growth of \textit{V. parahaemolyticus} will not occur in 1% peptone water in the absence of NaCl; however, growth could be obtained in this medium with either 3 or 7% NaCl.

Baross and Liston (1970), in a comparison of Japanese strains of \textit{V. parahaemolyticus} to isolates obtained from Puget Sound, reported that all of the test strains from both sources grew in the presence of 7.0% NaCl. However, three
Puget Sound strains grew at 10.0% salt while none of the 40 Japanese strains grew at that concentration. None of these percentages were related to $a_w$ values.

Troller (1979) described several enterobacteriaceae (Klebsiella, Proteus, Citrobacter, Shigella and Escherichia) which grew at minimum $a_w$ ranges of 0.94-0.96. The data presented was averaged from several workers; the humectant used was not supplied.

The fact that \textit{Y. enterocolitica} is classified in the same family as these organisms leads one to expect growth of \textit{Y. enterocolitica} at these $a_w$ ranges when all other factors (temperature, pH) are optimum.
MATERIALS AND METHODS

Test Cultures

The three strains of \textit{Y. enterocolitica} studied were: ATCC 27739 (serotype 0:8, from street water, typical) obtained from American Type Culture Collection; 1049 and 1059 (serotype 0:16 and 0:22 respectively, from vacuum packaged beef and lamb, atypical) obtained from M. O. Hanna, Texas A&M University. These strains were selected in order to compare typical isolates versus atypical isolates and a common human serotype versus serotypes isolated from a food source.

Media

The following media were used during the course of this investigation. (i) Brain heart infusion broth + 1.5% agar (BHIA) was employed to maintain stock cultures (held at 4C and transferred tri-monthly) and to obtain total counts. (ii) Brain heart infusion broth (BHI) was employed for water activity (a$_w$) studies at elevated salt levels, determination of inocula and determination of growth curves. (iii) Brain heart infusion broth + 1.5% salt (BHIS) was employed as the basal medium for a$_w$ studies involving the solutes sucrose and glycerol. All media were prepared with BHI from the same production lot.
Biochemical Evaluation of Test Strains

Each strain was inoculated into 10 ml of BHI broth and incubated for 24 h at 25 and 37°C. Following incubation, each culture was centrifuged, the supernatant decanted and the pellicle resuspended in 10 ml of sterile distilled water. All three strains were inoculated into the following conventional media: 1% tryptone broth, motilityornithine decarboxylase medium (M-O) (Ederer, Clark and Clark, 1970), Christensen urea slants, MR-VP broth, Simmons citrate, triple sugar iron agar slants (TSI) and lysine decarboxylase semisolid medium. Beta-galactosidase was detected from TSI slants (Food and Drug Administration, 1976). One percent solutions of the following carbohydrates were individually sterilized in phenol red broth base (Difco): raffinose, glucose, salicin, rhamnose, melibrose and arabinose. Inoculated media were incubated and reactions read at 2, 4 and 7 days. Kovac reagent was used to detect the presence of indole in the tryptone tubes. Differential charts presented in Darland, Ewing and Davis (1974) and Brenner et al. (1976) were used to identify test strains. Biochemical characteristics are presented in Table 2.

Determination of Inocula

One hundred ml of BHI were inoculated with 1 ml of a 20-24 h culture of each strain and incubated at 25°C for 24 h. Following incubation, cultures were centrifuged at 3,000 x g in a Sorvall RC2-B centrifuge for 10 minutes.
Table 2. Biochemical Evaluation of *Yersinia* enterocolitica
Serotypes 0:8, 0:17 and 0:22

<table>
<thead>
<tr>
<th>Biochemical Test</th>
<th>0:8</th>
<th>0:17</th>
<th>0:22</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25C</td>
<td>37C</td>
<td>25C</td>
</tr>
<tr>
<td>indole</td>
<td>+a</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>motility (M-O)</td>
<td>+</td>
<td>-b</td>
<td>+</td>
</tr>
<tr>
<td>TSI</td>
<td>A/A</td>
<td>A/A</td>
<td>A/A</td>
</tr>
<tr>
<td>urea</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ornithine decarboxylase (M-O)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>methyl red</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>lysine decarboxylase</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Voges-Proskauer</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>beta-galactosidase</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>citrate</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>glucose</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>raffinose</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>salicin</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>rhamnose</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>melibrose</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>arabinose</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

a. rapid reaction 24-48 h

b. negative reaction
The supernatant was decanted, and the pellet was resuspended in 20 ml of 0.1 M phosphate buffer, pH 7.0. The suspension of each strain was diluted to an absorbancy of 1.0 at 420 nm using a Bausch and Lomb Spectronic-20 colorimeter. Viable cell counts of the adjusted suspension were made on triplicate BHIA spread plates with sterile bent glass rods used to spread the inoculum. Following incubation at 25°C for 24 h, viable cell counts were determined from plates containing 30 to 300 colonies. Counts obtained for each strain were used to estimate cell population in the adjusted phosphate buffer suspension in order that a consistent inocula of approximately 10,000 cells/ml could be obtained. This method was used throughout this study.

**Growth Curves**

Growth curves were constructed for each strain. These data were collected in order to compare growth in BHI ($a_w = 0.996$, pH 6.8) to growth at lowered $a_w$, pH and temperature levels.

Inocula were prepared for each strain as previously described. Three 500 ml Erlenmeyer flasks containing 100ml of BHI each, were inoculated (initial population approximately $1 \times 10^4$ cells/ml) separately. Flasks were incubated at 25°C. Turbidity readings (Bausch and Lomb Spectronic - 20 colorimeter, 420 nm) and total counts (BHIA) were taken at 0, 3, 6, 9, 12, 18, and 24 h. The spread plate method using bent glass rods was employed for total counts. Duplicate
plates were incubated at 25°C for 48 h. A control flask was used for $a_w$ determination and as a blank for turbidity readings.

**Experimental Design**

For all experiments, the growth of each strain of *Y. enterocolitica* was periodically monitored by turbidity readings (Bausch and Lomb Spectronic-20 colorimeter, 420 nm). The magnitude of growth is expressed as optical density units. Initial and final total counts of viable cells were made (BHIA) using the spread plate method. Plates were incubated at 25°C for 48 h. These counts are expressed as number of viable cells/ml. This was done in order to determine cell survival, death and maximum populations. Maximum population is defined as total viable cells at the termination of incubation. Survival is defined as a final total count of less than initial count. Death is defined as a final total count of less than ten per ml.

Growth media, adjusted to various $a_w$ levels and pH levels, were dispensed in 10 ml amounts into test tubes (18 x 150 mm test tube; Corning). Tubes for each test were then inoculated (as previously described), plugged (DISPO, Scientific Products) and incubated at either 25°C for 10 days or 4°C for 21 days. Once a minimal $a_w$ level was determined, (for each strain in each solute; NaCl, sucrose, glycerol) the pH was adjusted at that level in order to observe additional inhibition on each strain due to pH. Incubation
temperatures were kept constant at 25°C and 4°C. The former incubation period was selected to give cells a reasonable amount of time to grow at optimum temperature under lowered $a_w$ and pH levels. The later was selected because previous workers (Hanna et al. 1976, Greenwood et al. 1975) have shown the need for long incubation periods at 4°C to reach maximum populations and 4°C is a common storage temperature for many refrigerated foodstuffs. Positive and negative control tubes and blanks for turbidity measurements and $a_w$ determination were prepared for each experiment.

**Adjustment of $a_w$**

Three experiments were designed to observe the response of each of three strains of *Y. enterocolitica* in media of progressively lowered $a_w$ levels. For each experiment, the $a_w$ adjustment was achieved with a different solute: NaCl, sucrose or glycerol. Media were routinely prepared within 24 h of use. No attempt was made to maintain a constant $a_w$ level following inoculation. All tubes were sealed with plastic plugs (as previously described) to reduce evaporation to a minimum.

For studies involving growth of the selected strains at elevated NaCl levels, BHI was the basal medium. When prepared according to the manufacturer's directions, BHI contains 0.5% NaCl. To elevate NaCl levels, quantities of NaCl were added to known volumes of BHI. The percentage of NaCl was expressed as follows:
Total grams of NaCl (grams NaCl added plus 0.5% NaCl in BHI) grams of final solution (grams NaCl added plus ml BHI)

Each of the remaining solutes studied (sucrose and glycerol) were added individually to BHIS. Percentage of these two solutes were calculated as follows:

\[
\text{Grams of solute} = \frac{\text{Grams of solute plus ml BHIS}}{\text{Grams of solute plus ml BHIS}}
\]

The three solutes were added in progressively increasing amounts to achieve water activity ranges (for each solute) that were expected to yield and inhibit growth of \textit{Y. enterocolitica}. Amounts of solutes added, desired solute percent, their calculated weight percents and resulting measured and corrected (from calibration line) \(a_w\) are summarized in Table 3.

**Adjustment of pH**

In determining the effect of interaction of variation in degree of acidity and \(a_w\) on the growth of \textit{Y. enterocolitica}, the media were adjusted as follows. Five normal NaOH or 6 N HCl was added in appropriate amounts to the respective unautoclaved BHI/NaCl or BHIS/glycerol of sucrose medium at the lowest percentage solute to yield growth to produce the desired pH value (+ 0.1 pH unit) after autoclaving. A Corning (model 125) pH meter was used to measure the pH of the respective media. The pH levels used (pH 5, 6 and 7) were most expected to be encountered in a foods system. Triplicate tubes, controls and appropriate blanks were prepared; tubes were incubated and growth monitored as previously described.
Table 3. Effect of Added Solutes on $a_w$ of BHI and BHIS

<table>
<thead>
<tr>
<th>Solute</th>
<th>g of solute added per 100 ml</th>
<th>Desired solute %</th>
<th>Calculated solute concentration (actual) (% wt.)</th>
<th>$a_w$</th>
<th>Measured</th>
<th>Corrected$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>2.08</td>
<td>2.50</td>
<td>2.52</td>
<td>0.971</td>
<td>0.971</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.85</td>
<td>3.25</td>
<td>3.25</td>
<td>0.959</td>
<td>0.959</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.25</td>
<td>4.50</td>
<td>4.56</td>
<td>0.949</td>
<td>0.949</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6.00</td>
<td>6.00</td>
<td>6.13</td>
<td>0.937</td>
<td>0.938</td>
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<tr>
<td></td>
<td>7.05</td>
<td>7.00</td>
<td>7.05</td>
<td>0.928</td>
<td>0.929</td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>14.3</td>
<td>12.50</td>
<td>12.51</td>
<td>0.946</td>
<td>0.946</td>
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<tr>
<td></td>
<td>33.3</td>
<td>25.00</td>
<td>24.98</td>
<td>0.936</td>
<td>0.936</td>
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<tr>
<td></td>
<td>43.0</td>
<td>30.00</td>
<td>30.07</td>
<td>0.921</td>
<td>0.922</td>
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<tr>
<td></td>
<td>100.0</td>
<td>50.00</td>
<td>50.00</td>
<td>0.875</td>
<td>0.876</td>
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<tr>
<td>Glycerol</td>
<td>15.0</td>
<td>13.00</td>
<td>13.04</td>
<td>0.944</td>
<td>0.944</td>
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</tr>
<tr>
<td></td>
<td>16.5</td>
<td>14.00</td>
<td>14.16</td>
<td>0.939</td>
<td>0.939</td>
<td></td>
</tr>
<tr>
<td></td>
<td>18.0</td>
<td>15.00</td>
<td>15.20</td>
<td>0.937</td>
<td>0.938</td>
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<tr>
<td></td>
<td>22.0</td>
<td>18.00</td>
<td>18.03</td>
<td>0.931</td>
<td>0.931</td>
<td></td>
</tr>
<tr>
<td></td>
<td>23.5</td>
<td>19.00</td>
<td>19.02</td>
<td>0.922</td>
<td>0.923</td>
<td></td>
</tr>
<tr>
<td></td>
<td>25.0</td>
<td>20.00</td>
<td>20.00</td>
<td>0.916</td>
<td>0.917</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ From calibration line
(experimental design). During growth of the three strains, no attempt was made to maintain a constant pH level.

Determination of $a_w$

Water activity readings were obtained through the use of a Beckman Sina-scope. The Sina-scope was equipped with a recorder which indicated when equilibrium had occurred between food, air space and sensor element. Because the volume of air space between the sensor was small, equilibrium was established rapidly, generally within one hour. The system calibration was expanded to measure $a_w$ over the range of 0.76 - 1.0. The Sina-scope sensor was equipped with a mechanical filter (protection from dust, oil and water vapor condensation) and a chemical filter (protection from volatiles such as glycerol).

The instrument was calibrated according to the method described by Stoloff (1977). Five salts (Analytical Reagent Grade, AR) were selected to cover $a_w$ range of sensor being used. A slurry of each salt was prepared with deionized water. The $a_w$ of each salt slurry was measured at 25°C and recorded. Instrument readouts for each slurry were plotted against theoretical $a_w$ values at 25°C reported by Labuza et al. (1976) using cross-section paper scaled for reading to 0.001 $a_w$ units. The best average linear line through the plotted points was constructed. This calibration line was used to correct direct instrument readouts. This method of calibration allows reporting of $a_w$ to three decimal places.
Theoretical $a_w$ values and measured $a_w$ values obtained for each salt slurry are given in Table 4. The calibration plot is shown in Figure 1.

The procedure for use of the Sina-scope was as follows:

1. A 5 ml sample was pipetted into a plastic sina dish after room temperature equilibration. The open dish was placed in the base and the sensor positioned, forming an air-tight seal. The base/sensor unit was placed in a styrofoam fox with a lid to decrease the effect of temperature fluctuation (Duckworth, 1975). The box was housed in a $25 \pm 0.1^\circ C$ incubator.

2. The indicator/recorder was activated, a one hour equilibration period observed and the percent relative humidity read directly from a bar scale on the indicator and corrected with the calibration line.

3. The $a_w$ was determined at the end of the incubation periods.
Table 4. Measured and Theoretical$^a$ $a_w$ Values of Salt slurries Used to Construct the Calibration Line for the Beckman Sina-Scope

<table>
<thead>
<tr>
<th>Salt Slurry</th>
<th>Theoretical $a_w$ @ 25C</th>
<th>Measured $a_w$ @ 25C</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>0.758</td>
<td>0.755</td>
</tr>
<tr>
<td>$(\text{NH}_4)_2\text{SO}_4$</td>
<td>0.803</td>
<td>0.801</td>
</tr>
<tr>
<td>KCl</td>
<td>0.843</td>
<td>0.840</td>
</tr>
<tr>
<td>KNO$_3$</td>
<td>0.920</td>
<td>0.919</td>
</tr>
<tr>
<td>$\text{K}_2\text{SO}_4$</td>
<td>0.969</td>
<td>0.970</td>
</tr>
</tbody>
</table>

$^a$ From Labuza et al. (1976).
Fig. 1. Plot of actual vs theoretical water activities for five salt slurries to construct the calibration line for the Beckman Sina-scope.
RESULTS

Effect of \( a_w \) and Temperature

The primary purpose of this phase of the study was to determine how the growth of \textit{Y. enterocolitica} serotypes 0:8, 0:17 and 0:22 was affected by reduced water activity levels. Behavior of an organism to reduced \( a_w \) levels also involves the solute which is selected to reduce \( a_w \). Therefore, three different solutes (NaCl, sucrose and glycerol) were employed to observe the further effect of type of solute on the growth of these three serotypes of \textit{Y. enterocolitica}. No attempt was made to adjust the \( a_w \) level to the same point with each solute. The secondary purpose of this phase was to show the effect of different solute concentrations on growth, not to define a minimal \( a_w \) for growth for each solute. This somewhat complicates comparison of growth for each serotype at each solute concentration; however, the degree of inhibition of each solute at varying concentrations is amply demonstrated.

Table 5 summarizes the growth of each serotype under optimal conditions. Each serotype was grown in BHI at pH 6.8, \( a_w \) 0.998 and incubated at 25°C. Maximum populations at this \( a_w \), pH and temperature were obtained so comparisons could be made between optimal and reduced growth conditions. Growth curves (not presented) for each serotype
Table 5. Growth Response (average log number of cells/ml) of *Yersinia enterocolitica* Grown in Brain Heart Infusion Broth (a\(_w\) = .998; pH = 6.8) Incubated at 25C

<table>
<thead>
<tr>
<th>Incubation Period (hr.)</th>
<th>0:8</th>
<th>0:17</th>
<th>0:22</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4.17</td>
<td>4.08</td>
<td>4.09</td>
</tr>
<tr>
<td>3</td>
<td>4.44</td>
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</tr>
<tr>
<td>12</td>
<td>7.39</td>
<td>7.12</td>
<td>7.12</td>
</tr>
<tr>
<td>18</td>
<td>9.00</td>
<td>8.94</td>
<td>8.80</td>
</tr>
<tr>
<td>24</td>
<td>8.99</td>
<td>8.90</td>
<td>8.95</td>
</tr>
<tr>
<td>30</td>
<td>8.93</td>
<td>8.86</td>
<td>8.91</td>
</tr>
</tbody>
</table>
are quite similar. Serotype 0:8 did, however, obtain a slightly increased rate of growth and maximum population compared to 0:17 and 0:22 under these conditions.

Response of each serotype to reduced $a_w$ adjusted with NaCl and incubated at 25 or 4C is presented in Table 6. As demonstrated by final populations, decreasing the $a_w$ to 0.971 with NaCl resulted in decreased maximal growth for each serotype at each temperature. Further lowering of the $a_w$ progressively decreased the level of maximal growth attained for each strain at both temperatures. Strain variation was demonstrated for growth at 4C and for $a_w$ level at which growth was obtained or survival occurred. The lowest $a_w$ level at which serotype 0:8 was able to grow was 0.949 for both temperatures. However, maximal populations were approximately 1.5 logs higher at 25C than 4C at this $a_w$. Survival was recorded for serotype 0:8 at $a_w$ 0.938. Surviving numbers were higher at 4C than 25C for $a_w$ levels 0.938 and 0.929.

Serotypes 0:17 and 0:22 had similar growth patterns at 25C. The lowest $a_w$ level at which these serotypes grew was 0.959 for 25C. Survival occurred at a lower $a_w$ (0.938) for 0:22 than for 0:17 (0.949) at 25C. The $a_w$ at which death was observed at 25C was higher (0.938) for 0:17 than 0:22 (0.928). Although death did occur at different $a_w$ levels for these two serotypes, no death was observed for serotype 0:8 at similar $a_w$ levels for either temperature.

At 4C, a similar pattern was observed for 0:17 and 0:22 which differed from 0:8. Maximum populations were higher.
Table 6. Final Log Average Population\(^a\) of \textit{Yersinia enterocolitica} Grown in BHI Adjusted to Varying \(a_w\) with NaCl and Incubated at 25\(^b\) or 4\(^c\) C

<table>
<thead>
<tr>
<th>Corrected(^d) (a_w) level</th>
<th>serotype 0:8</th>
<th>serotype 0:17</th>
<th>serotype 0:22</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25°C</td>
<td>4°C</td>
<td>25°C</td>
</tr>
<tr>
<td>0.971</td>
<td>8.51</td>
<td>7.89</td>
<td>8.28</td>
</tr>
<tr>
<td>0.959</td>
<td>8.16</td>
<td>7.49</td>
<td>7.42</td>
</tr>
<tr>
<td>0.949</td>
<td>7.00</td>
<td>5.83</td>
<td>2.66</td>
</tr>
<tr>
<td>0.938</td>
<td>3.03</td>
<td>3.83</td>
<td>&lt;1.00</td>
</tr>
<tr>
<td>0.929</td>
<td>1.42</td>
<td>3.71</td>
<td>&lt;1.00</td>
</tr>
</tbody>
</table>

\(^a\) Initial population for each strain was ca log\(_{10}\) 4.00 cells/ml. Values presented represent log\(_{10}\) of number of cells/ml in triplicate.

\(^b\) 10 day incubation

\(^c\) 21 day incubation

\(^d\) From calibration line
at 4C than at 25C. However, growth for 0:17 was recorded at a higher $a_w$ level (0.959) than 0:22 (0.949). The $a_w$ level at 4C for 0:22 was the same as that observed for serotype 0:8. Survival of 0:17 and 0:22 at 4C was the same as 0:8 (0.929). No death was observed for any of the strains at 4C for any $a_w$ level.

Although maximum populations for each serotype varied at the two higher $a_w$ levels, survival was greater at 4C for each serotype at $a_w$ 0.938 and 0.929. The data indicates that 4C reduces the rate of cellular fluid transfer through membranes creating a metabolic slowdown which contributes to the survival of these serotypes at $a_w$ levels lowered with NaCl.

The effect of $a_w$ reduced with sucrose upon growth of *Y. enterocolitica* at 4C and at 25C is summarized in Table 7. Sucrose was not as inhibitory to the organism as NaCl at 25C. As the $a_w$ level was reduced to 0.946, maximum growth was reduced from that attained at 0.998 (BHI broth) but were significantly higher than maximum populations observed at a similar $a_w$ (0.949) adjusted with NaCl.

At 25C, no growth was observed for the three serotypes below 0.936. Survival of the organism showed some strain variation at different $a_w$ levels at the higher temperature. Serotype 0:17 was least resistant to lowered $a_w$ levels adjusted with sucrose. Death was observed at 0.936 and lower $a_w$ levels at 25C. Death was not recorded for serotype 0:8.
Table 7. Final Log Average Population\(^a\) of Yersinia enterocolitica Grown in BHIS Adjusted to Varying \(a_w\) with Sucrose and Incubated at 25\(^b\) or 4C\(^c\)

<table>
<thead>
<tr>
<th>Corrected(^d) (a_w) level</th>
<th>serotype 0:8 (25^\circ C)</th>
<th>4C</th>
<th>serotype 0:17 (25^\circ C)</th>
<th>4C</th>
<th>serotype 0:22 (25^\circ C)</th>
<th>4C</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.946</td>
<td>8.73</td>
<td>4.95</td>
<td>8.63</td>
<td>3.20</td>
<td>8.53</td>
<td>4.11</td>
</tr>
<tr>
<td>0.936</td>
<td>2.22</td>
<td>3.94</td>
<td>&lt;1.00</td>
<td>3.58</td>
<td>1.22</td>
<td>4.45</td>
</tr>
<tr>
<td>0.922</td>
<td>&lt;1.00</td>
<td>4.70</td>
<td>&lt;1.00</td>
<td>4.47</td>
<td>1.74</td>
<td>3.58</td>
</tr>
<tr>
<td>0.876</td>
<td>&lt;1.00</td>
<td>3.51</td>
<td>&lt;1.00</td>
<td>1.72</td>
<td>&lt;1.00</td>
<td>3.71</td>
</tr>
</tbody>
</table>

\(^a\) Initial population for each strain was ca \(\log_{10} 4.00\). Value presented represents \(\log_{10}\) of number of cells/ml in triplicate.

\(^b\) 10 day incubation

\(^c\) 21 day incubation

\(^d\) From calibration line
until the $a_w$ level was reduced to 0.922. Serotype 0:22 was able to survive at $a_w$ 0.022 at 25°C.

Response to $a_w$ levels adjusted with sucrose at 4°C was similar for each serotype. The only serotype able to generate growth at 0.946 at the lower temperature was 0:8. The same metabolic slowdown observed with NaCl seemed to be evident with sucrose. Survival of all three serotypes was observed at the lowest $a_w$ achieved with sucrose (0.876). Survival populations were somewhat higher when the $a_w$ was adjusted with the sucrose than with NaCl at a similar $a_w$: 0.922 versus 0.929, respectively.

Data generated for effect of $a_w$ when the $a_w$ levels were obtained by adjustment with glycerol are presented in Table 8. A much wider range of $a_w$ levels were found to support growth depending on incubation temperature. Strain variation was again observed for $a_w$ level to support growth and temperature effect.

The atypical strains, 0:17 and 0:22, were not able to grow at an $a_w$ level as low as 0:8 when incubated at 4°C. Serotype 0:8 was able to grow at an $a_w$ of 0.939 whereas 0:17 and 0:22 were only able to generate growth at the highest $a_w$ level tested (0.944). Survival of the three serotypes also showed a variation between the atypical strains and the typical strain. Survivors were detected for 0:17 and 0:22 at $a_w$ 0.939 (0:8 grew at this level); however, 0:8
Table 8. Final Log Average Population\textsuperscript{a} of \textit{Yersinia enterocolitica} Grown in BHIS Adjusted to Varying $a_w$ with glycerol and Incubated at 25\textsuperscript{b} or 4\textsuperscript{C}

<table>
<thead>
<tr>
<th>Corrected\textsuperscript{d} $a_w$ level</th>
<th>serotype 0:8</th>
<th>serotype 0:17</th>
<th>serotype 0:22</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25\textsuperscript{C}</td>
<td>4\textsuperscript{C}</td>
<td>25\textsuperscript{C}</td>
</tr>
<tr>
<td>0.944</td>
<td>-\textsuperscript{e}</td>
<td>7.95</td>
<td>-</td>
</tr>
<tr>
<td>0.939</td>
<td>-</td>
<td>5.97</td>
<td>-</td>
</tr>
<tr>
<td>0.938</td>
<td>-</td>
<td>3.41</td>
<td>-</td>
</tr>
<tr>
<td>0.931</td>
<td>6.50</td>
<td>-</td>
<td>7.80</td>
</tr>
<tr>
<td>0.923</td>
<td>6.00</td>
<td>-</td>
<td>&lt;1.00</td>
</tr>
<tr>
<td>0.917</td>
<td>1.77</td>
<td>-</td>
<td>&lt;1.00</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Initial population for each strain was ca $\log_{10} 4.00$. Value presented represents $\log_{10}$ of number of cells/ml in triplicate.

\textsuperscript{b} 10 day incubation

\textsuperscript{c} 21 day incubation

\textsuperscript{d} From calibration line

\textsuperscript{e} Not tested

1
survived at 0.938. No death was observed for any serotype at any $a_w$ level tested when incubated at 4C. No testing was done at 4C below an $a_w$ of 0.938 due to no growth of any serotype below $a_w$ 0.939.

A definite temperature effect was noted when glycerol was the solute employed. Growth of each serotype was obtained at significantly lower $a_w$ levels when the incubation temperature was increased to 25C. All three serotypes were able to grow at an $a_w$ of 0.931. An unexpected strain variation was noted at this point. Serotypes 0:8 and 0:22 were able to grow at $a_w$ 0.923 while death was observed for 0:17 at this level. Death was not observed for 0:22 until the $a_w$ was reduced to 0.917. Survival of 0:8 was noted at $a_w$ 0.917 at 25C.

**Effect of Solute**

The particular solute used to reduce $a_w$ level of the basal medium appeared to affect the magnitude of growth of each strain and the $a_w$ level which permitted growth. Sucrose and NaCl were more inhibitory than glycerol at either temperature.

At 25C, growth of serotype 0:8 occurred at an $a_w$ of 0.949 and 0.946 for NaCl and sucrose, respectively. When glycerol was employed as the solute, the $a_w$ level which supported growth was reduced to 0.923. Not as dramatic an effect was observed between solutes at 4C as was observed at 25C for serotype 0:8. The $a_w$ levels at which growth
occurred at 25C were the same at 4C for sucrose and NaCl; however, O:8 was able to grow at a lower \( a_w \) level (0.939) when glycerol was the solute. These data are illustrated in Figure 2.

A similar effect of solute was observed for the atypical strains. When NaCl was the solute, at 25C, growth of O:17 and O:22 was not observed below an \( a_w \) of 0.959. Sucrose produced the same effect as was observed for serotype O:8. Again, much less inhibition was demonstrated with glycerol. However, O:17 was unable to grow at the same \( a_w \) level that allowed O:8 and O:22 to grow (0.923). The \( a_w \) for O:17 for glycerol was 0.931. These data are summarized in Figures 3 and 4.

When NaCl was the solute employed, no further inhibition of growth for the three serotypes was observed even though the incubation temperature was reduced to 4C. The \( a_w \) levels permitting growth of each serotype were the same as those described for 25C. Sucrose and glycerol, however, demonstrated further inhibition at the lower temperature. For the atypical strains, sucrose inhibited growth at the highest level tested (0.946). Growth for serotype O:8 at this level was greatly reduced at 4C and only slight growth was observed. Glycerol, again, was the less inhibitory of the three solutes tested. Serotype O:8 was able to initiate growth at an \( a_w \) of 0.939. The two atypical strains were able to initiate growth at a slightly higher \( a_w \) of 0.944. These
Fig. 2. Combined Effect of Temperature, pH and $a_w$ (adjusted to lowest level to yield growth for each respective solute) on the Maximum Population of *Yersinia enterocolitica* serotype 0:8.-- Values presented represent a triplicate average. Maximum population defined as turbidity at end of 10 days incubation for 25°C and 21 days at 4°C.
Fig. 3. Combined Effect of Temperature, pH and $a_w$ (adjusted to lowest level to yield growth for each respective solute) on the Maximum Population of *Yersinia enterocolitica* serotype 0:17. — Values presented represent a triplicate average. Maximum population defined as turbidity at end of 10 days incubation for 25°C and 21 days at 4°C.
Fig. 4. Combined Effect of Temperature, pH and $a_w$ (adjusted to lowest level to yield growth for each respective solute) on the Maximum Population of Yersinia enterocolitica serotype 0:22. — Values presented represent a triplicate average. Maximum population defined as turbidity at end of 10 days incubation for 25°C and 21 days at 4°C.
aw levels are significantly lower than those established for sucrose and NaCl.

Effect of pH

The purpose of this portion of the study was to observe how pH further inhibited growth at the lowest solute concentration which previously permitted growth. The pH levels tested were 7.0, 6.0 and 5.0. Each of the lowest solute concentrations (aw level) was adjusted to three different pH levels. Again, two incubation temperatures (4C or 25C) were used for each aw/pH combination. These pH levels are representative of the range most often encountered in food systems.

Table 9 summarizes data for the combined effect of solute concentration (aw) and pH at 25C. As the pH value of the growth medium was lowered, growth response of each serotype continued, but at a level which seemingly was influenced by the degree of acidity in the environment. Although the growth response to different pH values within each aw level, and to the aw levels adjusted by each solute, varied with respect to maximal growth attained, the pattern for each serotype was essentially the same.

At any aw level (for each solute) at which growth occurred in media adjusted to pH 7.0, growth also occurred in media of pH 6.0.
Table 9. Final Log Average Population\textsuperscript{a} of *Yersinia enterocolitica* Grown in Basal Media\textsuperscript{b} Adjusted to Varying pH Values at Minimal $a_w$ to Yield Growth for Each Solute Incubated at 4°C

<table>
<thead>
<tr>
<th>Minimal corrected\textsuperscript{d}</th>
<th>serotype 0:8</th>
<th></th>
<th></th>
<th>serotype 0:17</th>
<th></th>
<th></th>
<th>serotype 0:22</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>$a_w$</td>
<td>pH 7.0</td>
<td>pH 6.0</td>
<td>pH 5.0</td>
<td>pH 7.0</td>
<td>pH 6.0</td>
<td>pH 5.0</td>
<td>pH 7.0</td>
<td>pH 6.0</td>
<td>pH 5.0</td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td>0.959</td>
<td>-\textsuperscript{e}</td>
<td>-</td>
<td>-</td>
<td>7.42</td>
<td>7.92</td>
<td>&lt;2.00</td>
<td>7.00</td>
<td>7.22</td>
<td>&lt;2.00</td>
</tr>
<tr>
<td></td>
<td>0.949</td>
<td>7.00</td>
<td>8.15</td>
<td>&lt;2.00</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sucrose</td>
<td>0.946</td>
<td>8.73</td>
<td>8.36</td>
<td>&lt;2.00</td>
<td>8.63</td>
<td>7.58</td>
<td>&lt;2.00</td>
<td>8.53</td>
<td>7.54</td>
<td>&lt;2.00</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.931</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>7.81</td>
<td>6.72</td>
<td>&lt;2.00</td>
<td>6.60</td>
<td>6.23</td>
<td>&lt;2.00</td>
</tr>
<tr>
<td></td>
<td>0.923</td>
<td>6.00</td>
<td>6.95</td>
<td>&lt;2.00</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Initial population for each strain was ca $\log_{10}$ 4.00. Value presented represents $\log_{10}$ of number of cells/ml in triplicate.

\textsuperscript{b} 10 day incubation

\textsuperscript{c} 21 day incubation

\textsuperscript{d} From calibration line

\textsuperscript{e} Not tested
It may be stated generally that pH 6.0 permitted as
great or greater magnitude of growth as did pH 7.0. Death
was observed for all three serotypes at all $a_w$ levels when
the pH was reduced to pH 5.0.

There was some indication that, at the lowest $a_w$
level, the consequences of the interaction between $a_w$ and
various pH values on maximal growth of the strains was reduced.
That is, the $a_w$ level seemed to be more important in deter­
mining the amount of growth than was the lowered pH value.
Not as great an effect for pH was observed as was expected.

Table 10 summarizes the same parameters as discussed
for Table 8 with the exception of a reduction in incubation
temperature to 4C. In this case, the combination of $a_w$/pH
was much more inhibitory at eh lower temperature. Maximum
populations were not as high for any serotype at the adjusted
$a_w$ and various pH levels as compared to 25C.

Sucrose appeared to be more inhibitory than NaCl or
glycerol for 0:8. No data was available for the atypical
strains and sucrose due to their failure to grow at the
highest sucrose level in preliminary experiments.

Survival was observed for all serotypes at pH
6.0 and 5.0 at each adjusted $a_w$ level. Serotype 0:22 was
able to grow at pH 6.0 when the $a_w$ level was adjusted with
glycerol.

Again, the metabolic slowdown (at 4C) discussed
seemed to be evident. A high rate of survival (only slightly
Table 10. Final Log Average Population\textsuperscript{a} of \textit{Yersinia enterocolitica} Grown in Basal Media\textsuperscript{b} Adjusted to Varying pH Values at minimal a\textsubscript{w} to Yield Growth for Each Solute Incubated at 4\textdegree C\textsuperscript{c}

<table>
<thead>
<tr>
<th>Minimal corrected\textsuperscript{d}</th>
<th>serotype 0:8</th>
<th>serotype 0:17</th>
<th>serotype 0:22</th>
</tr>
</thead>
<tbody>
<tr>
<td>aw</td>
<td>pH 7.0</td>
<td>pH 6.0</td>
<td>pH 5.0</td>
</tr>
<tr>
<td>NaCl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.959</td>
<td>-\textsuperscript{e}</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.949</td>
<td>5.83</td>
<td>3.95</td>
<td>3.92</td>
</tr>
<tr>
<td>Sucrose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.946</td>
<td>4.95</td>
<td>4.44</td>
<td>4.23</td>
</tr>
<tr>
<td>Glycerol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.944</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.939</td>
<td>5.97</td>
<td>4.62</td>
<td>4.27</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Initial population for each strain was ca $\log_{10} 4.00$. Value presented represents $\log_{10}$ of number of cells/ml in triplicate.

\textsuperscript{b} 10 day incubation

\textsuperscript{c} 21 day incubation

\textsuperscript{d} From calibration line

\textsuperscript{e} Not tested
reduced from inocula) of each serotype was observed at pH 6.0 and 5.0. This held true even though $a_w$ was at or near a limiting value. The survival at pH 5.0 was unexpected even with the metabolic slowdown taken into consideration.
DISCUSSION

The purpose of this study was to observe the combined effect of several environmental parameters on the growth of *Yersinia enterocolitica* serotypes 0:8, 0:17 and 0:22. Little work has been done with this organism concerning growth parameters encountered in foods. The parameters selected for this study were water activity (aw), pH and incubation temperature. No published work involving effect of aw on *Y. enterocolitica* is presently available. Therefore, this parameter was chosen as the primary subject of this study. Temperature and pH were added, in combination, to further observe the inhibition on the growth of *Y. enterocolitica* at low aw levels.

Due to the fact that no previous work has been published on limiting aw for *Y. enterocolitica*, several random levels in increasing amounts of three different solutes were used to show the effect of aw. Duckworth (1975) states that more than one solute should be tested in order that effect of solute used to adjust aw can be considered when establishing limiting aw levels for growth. The three solutes were selected on the basis of an electrolyte (NaCl), a nonelectrolyte (glycerol) and a sugar (sucrose).

The relationship between limiting aw levels for growth of micro-organisms and the solute added to achieve
those levels is unclear. Several authors (Emodi and Lechowich 1969, Scott 1957) have stated that biological response to $a_w$ by some organisms is independent of the types of solutes used to reduce the $a_w$. Other reports have shown that nutrient availability (Christian 1955a), pH and moisture content (Plitman et al. 1973), in addition to the test solute, effect a micro-organism's ability to grow at limiting $a_w$. The evidence accumulated in the present study would suggest that the specific solute used to reduce the $a_w$ of the medium has a bearing on the limiting $a_w$ level required for the growth of the serotypes tested.

Addition of solutes to the medium reduced $a_w$ in all cases, but the magnitude of change in growth parameters was varied, depending upon the solute added. Glycerol had the least inhibitory effect on all three serotypes. The possibility that glycerol is a nutrative solute should not be ignored. The fact that glycerol does not alter osmotic pressure to as great an extent as NaCl and sucrose may also explain the lower $a_w$ level to allow growth when glycerol was the solute. The glycerol data are in agreement with those reported for *Staphylococcus aureus* survival (Plitman et al. 1973) and enterotoxin production (Troller 1971) and growth of *Salmonella oranienburg* (Christian 1955b), *Bacillus cereus* (Jakobsen, Filtenborg and Bramsaes 1972) and *Clostridium perfringens* (Kang et al. 1969), wherein glycerol was found to be less inhibitory than other test solutes. No
data are presently available to compare with the results of this study for *Y. enterocolitica* relative to $a_w$ and growth. Examples of some limiting levels for organisms of the same family have been previously discussed in the literature review.

Alteration of the environment, once the lowest $a_w$ level to allow growth was established, by increasing acidity and reducing incubation temperature had a further inhibitory effect. Wodzinski and Frazier (1960, 1961a, 1961b) have reported that, in their investigations with *Pseudomonas fluorescens*, *Aerobacter aerogenes* and *Lactobacillus viridescens* at nonoptimal pH and temperature levels for growth, the organisms were less tolerant to low $a_w$ levels; that is, as the environment moved further away from the optimal conditions, the minimal $a_w$ necessary for growth was increased.

Results obtained in this study tend to agree with those results. Temperature appeared to be more of an environmental factor than did pH. The $a_w$ level which permitted growth was raised for all three serotypes when the incubation temperature was lowered to 4°C and the solute used was glycerol. At 4°C, the highest $a_w$ level, adjusted with sucrose, was completely inhibitory to serotype 0:17 and 0:22 and only allowed slight growth of 0:8. The atypical strains demonstrated a greater resistance to the lower temperature and $a_w$ with NaCl as the solute. This may be due to the fact
that 0:8 was much closer to its limiting aw than the 0:17 and 0:22 serotypes.

The pH level had varying effects on growth depending on the incubation temperature and the solute used to reduce aw. A few general summaries may be made about the effect of pH. At 25°C, when growth occurred in media adjusted to pH 7.0, growth also occurred at pH 6.0. When the pH level was reduced to 5.0, death was observed for each serotype. At the lower temperature, pH 6.0 did not allow growth except for the atypical strains at an aw of 0.959 (NaCl) and serotype 0:22 at an aw of 0.944 (glycerol). In these cases, maximal populations were much reduced as compared to pH 7.0. The unexpected data obtained here was the high survival rate of each serotype at each solute even though the pH was reduced to 5.0. As previously discussed, there is a biological slowdown at the lower temperature which permits higher surviving numbers.

These data plus the fact that Y. enterocolitica is able to grow at 4°C, indicate this organism may constitute a significant public health hazard. These data suggest even reduced pH and refrigeration temperatures are ineffective for destruction of Y. enterocolitica. Upon product abuse (usually increased storage temperatures), these surviving organisms may grow in foods and attain levels which present a public health problem.
Response to $a_w$, pH, solute and temperature demonstrated a definite strain variation. Generally, the atypical strains were somewhat less resistant to reduction of optimal conditions. Variations between atypical and typical strains are documented and results obtained in this study are in agreement.

The effect of $a_w$ on the growth of *Y. enterocolitica* shown in this study agrees with observations made by several workers on the relationship between other micro-organisms and their moisture requirements. Inherent in a consideration of limiting $a_w$ levels is the whole spectrum of microbial environment, especially the pH level and temperature of incubation. The significance of these factors lies in the fact that food provides an environment which combines various degrees of $a_w$ and pH. In general, food is a more complex substrate than a liquid medium in terms of microenvironments created by physical structure and nutrient content. These characteristics of food may provide protection to the organism from adverse environmental conditions. Data from this study suggest that this protection is further enhanced for *Y. enterocolitica* when incubation is reduced to refrigeration temperatures. The temperature at which the food is held, the pH and $a_w$ levels of the food all become important factors in determining the ability of an organism to grow in that particular food.
Data generated in the present study do not define limiting $a_w$ for each strain. However, some idea of limiting $a_w$ levels for three different solutes have been established. The data generated does, however, permit predictions of types of products which could allow growth of these serotypes. Table 11 lists several common products with various $a_w$ levels, storage conditions and pH combinations. An estimation of the probability of growth of these serotypes can be made using the data obtained in this study.

Further inhibition by pH and temperature at lower $a_w$ levels has also been demonstrated. Further experiments are required to establish more closely the limiting $a_w$ of this organism. It appears that these serotypes of Y. enterocolitica are somewhat more tolerant of reduced $a_w$ levels than other similar Gram-negative organisms. This, coupled with its ability to grow at refrigerated temperatures and documented public health significance justify the need for further work involving the effects of various food processing and storage parameters on the growth of Y. enterocolitica.
Table 11. An Estimation of the Probability of Growth of *Yersinia enterocolitica* serotypes 0:8, 0:17 and 0:22 in Products with Various Water Activity ($a_w$), Storage Conditions and pH Values

<table>
<thead>
<tr>
<th>Product</th>
<th>Typical $a_w$</th>
<th>Typical pH</th>
<th>Typical Storage Condition</th>
<th>Probability of Growth $^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hot Dog</td>
<td>0.970</td>
<td>6.0</td>
<td>Refrigerated</td>
<td>Fair</td>
</tr>
<tr>
<td>Fresh Meat</td>
<td>0.990</td>
<td>6.5</td>
<td>Refrigerated</td>
<td>Good</td>
</tr>
<tr>
<td>Dry Sausage</td>
<td>0.910</td>
<td>5.0</td>
<td>Refrigerated/Room Temperature</td>
<td>None</td>
</tr>
<tr>
<td>Cheddar Cheese</td>
<td>0.940</td>
<td>5.4</td>
<td>Refrigerated</td>
<td>Poor</td>
</tr>
<tr>
<td>Ham</td>
<td>0.920</td>
<td>5.8</td>
<td>Refrigerated</td>
<td>Poor</td>
</tr>
<tr>
<td>Semi-moist Pet Food</td>
<td>0.800</td>
<td>6.0</td>
<td>Room Temperature</td>
<td>None</td>
</tr>
</tbody>
</table>

$^a$ Duckworth (1975)

$^b$ Does not account for possibility of survival


Leistner, L. and Rodel, W. 1976. Inhibition of microorganisms in food by water activity. SAB Symposium Series No. 5.


