OUTGROWTH OF TYPE E CLOSTRIDIUM BOTULINUM AS AFFECTED BY VARIOUS SOLUTES IN THE MEDIUM AND BY INCUBATION TEMPERATURE

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ABSTRACT

OUTGROWTH OF TYPE E *CLOSTRIDIUM BOTULINUM* AS AFFECTED BY VARIOUS SOLUTES IN THE MEDIUM AND BY INCUBATION TEMPERATURE

by Alexander Emodi

The objective of this study was to investigate the effects of the addition of various concentrations of solutes to one basic medium formulation upon the outgrowth of the spores of Type E *Clostridium botulinum* at different incubation temperatures.

Four strains, K, VH, SF, and A6247 were studied. An inoculum of 1.0×10^6/ml was inoculated into trypticase-peptone-sucrose-yeast extract (TPSY) medium and additions of sucrose, sodium chloride (NaCl), potassium chloride (KCl), glycerol and sodium formate were used. Equilibrium relative humidity (ERH) determinations were performed to determine the water activity (a_w) of the media. The possibility of an osmotic effect was also investigated. The molar concentrations and osmotic pressures of the different solutes were calculated and related to the approximate inhibitory concentrations of 38.5% sucrose (1.12 M), 5.0% NaCl (0.86 M), 6.0% KCl (0.80 M), and 5.5% sodium formate (0.80 M). No inhibition of growth was observed with the 15% (1.63 M) glycerol concentration. The use of 15% glycerol lowered the a_w but

did not inhibit outgrowth as observed with the other solutes.

Samples were incubated at 30, 21.1, 15.6, 10 and 7.2 C temperatures. Growth was observed by production of turbidity and gas. Toxicity was checked with the mouse toxin assay. Only those tubes which showed gas and gave a positive toxicity test were considered positive for Type E outgrowth. The inhibitory a_w level in these solutions appears to be between 0.9708 and 0.9755.

The effects of heat and the size of inoculum on outgrowth were also studied. Viable spore inocula of 1.0×10^2/ml, 1.0×10^4/ml and 1.0×10^6/ml of the K and VH strains were tested with and without heat shock in the various media. Spore suspensions were heat shocked for 15 minutes at 60 C. The size of the inoculum affected both lag and outgrowth times. Increased lag times and some growth inhibition were observed using 1.0×10^2 spores/ml at concentrations not inhibitory for the larger inoculum sizes.

Heat shock in most cases delayed or inhibited outgrowth at concentrations where spores without heat shock grew well. This adverse effect of the heat treatment was best demonstrated with the smaller 1.0×10^2/ml and 1.0×10^4/ml inoculum sizes. However, there was some indication with the VH strain that heat shock may permit outgrowth at concentrations previously found inhibitory for growth, and that the beneficial or adverse effect of the heat treatment depends very much on the strain involved.

Incubation temperature and growth response were inversely related as measured by increased lag times and in

decreased amounts of cellular material produced by each of the four Type E strains tested.

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By

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INTRODUCTION

Microorganisms, as living cells, require water for metabolism and growth. The water requirement of bacteria may be described with the water activity concept since water activity is a useful measure of the "availability" of water for microbial growth.

When substances are dissolved in water or bacteriological media there is a substantial interaction between solute and solvent. The extent to which the water activity is lowered depends primarily on the total concentration of all dissolved molecules and ions. Potassium chloride, sodium formate, sodium chloride and sucrose are very effective solutes to both lower water activity and change the osmotic pressure when added to bacteriological media.

In this study, the effects of water activity and osmotic phenomena on Type E Clostridium botulinum strains were examined using the variables of different solutes, incubation temperature, size of inoculum, and effect of preheating of the inoculum.

LITERATURE REVIEW

It is now widely accepted that the water requirements for growth of many microorganisms can be defined in terms of the water activity of their environment. In the present context the measurement of the equilibrium relative humidity (ERH), the control of ERH, and some of its effects upon the growth of Clostridium botulinum Type E strains will be discussed. The effects of certain solutes upon possible osmotic phenomena were also examined.

Definition of Water Activity

The availability of water has been expressed in different ways. Scott (1957, 1961) defined water activity or a_w as a fundamental property of aqueous solutions that is equal to:

$$\frac{p}{p_o} = \frac{n_2}{n_1+n_2}$$

where p is the vapor pressure of the solution and p_o is the vapor pressure of the solvent, and n_1 and n_2 refer to the number of moles of solute and solvent in the solution. Since water contains 55.51 moles/kg, for a 1.0 molal solution of an ideal solute the right-hand side of this equation will equal 55.51/56.51 = 0.9823. The a_w also is numerically equal to the corresponding relative humidity expressed as percentage divided by 100. Mossel and van Kuijk (1955) expressed

availability of water in terms of equilibrium relative humidity (h) which is numerically equal to a_w.

Description of a_w Limits for Growth of Microorganisms

Microbial growth has been observed by Frazier (1958), at a_w levels ranging from very close to 1.00 down to about 0.62.

Christian (1963) discussed the concept of a_w in relation to the water activities of foods and the a_w limits of the microorganisms involved in food spoilage and food poisoning.

Molds differ considerably among themselves as to optimum a_w and range of a_w for germination of the asexual spores. The minimum a_w for spore germination has been found to be as low as 0.62 for some molds, and as high as 0.93 for others.

Yeasts require more moisture than molds and on the basis of a_w may be classified as ordinary if they do not grow in high concentrations of solutes, that is, a low a_w, and as osmophilic if they do. Lower limits of a_w range from 0.88 to 0.94 for ordinary yeasts and from 0.62 to 0.75 for osmophilic yeasts.

Most bacteria grow well in the 0.995 to 0.998 a_w range. The lowest reported limit for growth is about 0.55.

General References on Water Activity and Microbial Growth

Walter (1924) found that none of the bacteria he tested could grow at relative humidities of less than 96%, whereas several molds grew at much lower humidity. Scott (1936, 1937) working with thin slices of beef muscle equilibrated with sulphuric acid solutions, found two strains of Achromobacter to have a lower limit for growth of approximately 96%, and two strains of Pseudomonas to have a lower limit greater than 98%. Numerous workers (Tomkins, 1929, Heintzeler, 1939 and Stille, 1948) working with molds agree that the greatest tolerance to low a_w is near the optimum growth temperature and that at lower and higher temperatures the range of a_w permitting germination and growth is reduced. Changes of 0.01 to 0.05 in the minimum a_w for a change of 10 C in incubation temperature have been noted. Similar data for bacteria have not been found.

Von Schelhorn (1950) noted that the greatest tolerance of Aspergillus glaucus to low a_w was manifest at the optimum pH, and that at lower and higher pH values the range of a_w permitting germination and growth was greatly reduced. Similar data for bacteria have not been reported.

Observation of staphylococci have shown that they generally tolerate somewhat higher solute concentrations than most other bacteria, and this has formed the basis of media used for enrichment and isolation of staphylococci as proposed by Chapman (1946).

In experiments with dried meat adjusted to various water contents, Segalove and Dack (1951) reported that a strain of Salmonella enteriditis required a greater amount of water for growth than Staphylococcus aureus or an alpha hemolytic-type Streptococcus.

Scott (1953) discussed the importance of the a_w in the relation to the growth of fourteen food poisoning strains of S. aureus. Aerobic growth was observed at a_w levels between 0.999 and 0.96. The rate of growth and the yields of cells were both reduced substantially when the a_w was less than 0.94. Anaerobic growth proceeded at slightly higher a_w than aerobic growth. He also showed that the reduction of the a_w below 0.99 led to an increase in the lag period.

Christian and Scott (1953) grew sixteen strains of Salmonella in various media of known water activities at 30 C. The reactions of fifteen motile strains were very similar, whereas the single non-motile strain grew more slowly and over a smaller range of water activities. Aerobic growth of the motile strains occurred in liquid media at water activities between 0.999 and 0.945. In food, the lower limit for growth was slightly less. Anaerobic rates of growth were only slightly less than the aerobic rate at all water activities. A large percentage of the cells could form colonies on agar media with water activities as low as 0.96.

There is some evidence for the belief that the water requirements of most coliform bacteria may be similar to those of the salmonellae. Foda and Vaughn (1950) found that 5 to 8% sodium chloride (NaCl) was needed to inhibit the growth of Escherichia coli. The 5 to 8% concentrations correspond to water activities of about 0.97 and 0.95 respectively. They also pointed out that most strains of Aerobacter aerogenes did not tolerate more than 6.5% NaCl (w/v), corresponding to 0.96 a_w.

Factors that Affect the Water Relationships of Microorganisms

Severens and Tanner (1945) studied the inhibition of growth by solutes such as NaCl, and reported that 3% NaCl was the highest concentration permitting growth of unadapted strains of Salmonella pullorum and Salmonella schottmuelleri (S. paratyphi B). After adaptation for about 10 weeks, growth was observed in media with 8% NaCl.

Christian and Scott (1953) found that the range of a_w over which growth occurred was substantially the same when sugar, salts or miscellaneous nutrients were the predominant solutes controlling the a_w.

Mossel and van Kuijk (1955) stated that the development of microbes in foods may be inhibited by appropriate desiccation or by addition of solutes such as sugar or salt in sufficient quantities.

A study of the growth of the Salmonella oranienburg in dried meat, dried milk and dried soup by Christian and Scott (1953) showed that at the limiting water activities

of 0.93 to 0.94, the water content of the soup was some three to four times as great as those of the milk and mutton. It is clear therefore that it is a_w rather than the water content which determines the availability of water for growth of these bacteria.

The water content of food gives no explicit information on ERH, as noted by Overbeek and Mossel (1951), since relatively small changes in the non-aqueous phase of biological material (e.g., partial replacement of cellulose by sugars or starch) may strongly influence its water binding capacity and hence its h value at a given water content.

Scott (1957) reported that the biological response to a particular water activity was, at least for some organisms, largely independent of the type of solute present and the total water content of the substrate.

The use of a_w as a measure of total solute concentration was employed by Wodzinski and Frazier (1960). The a_w of the medium was adjusted by the use of potassium chloride (KCl). Since sucrose alone and a salt mixture of NaCl, KCl, and sodium sulfate (Na_2SO_4) were just as effective in adjusting the a_w as KCl, it was evident that the effects observed were not due to either the concentration of the K ion or of the Cl ion. Both of these ions had a much lower concentration in the salts mixture than in KCl alone. These observations further insure that the responses observed were a function of a_w and not of the concentration of a particular solute or solutes.

Further support that the minimum a_w permitting growth changes with environmental conditions is found in the report of Bowling and Ramsey (1958). They showed that the combined effects of salts and chloramphenicol on Staphylococcus pyogenes were additive with most concentrations tested and frequently were synergistic.

Christian and Scott (1953) showed that various basal media supported the growth of several strains of salmonellae at water activities down to about 0.945. When observations were extended (1955) to a simple medium of inorganic salts and glucose, the expected decrease in growth rate at the optimum a_w was accompanied by a considerable reduction in the range of water activities permitting growth. This result indicated that growth of water activities of the order of 0.95 to 0.96 could only be realized when certain nutrients, absent from the simple medium, were provided.

It is generally agreed that most of the microorganisms tolerate low a_w at temperatures and pH levels most suitable for them as indicated by Ingram (1957) and Scott (1957). This is further supported in studies by Wodzinski and Frazier (1960, 1961). They investigated the water requirement for growth of A. aerogenes, Lactobacillus viridescens and Pseudomonas fluorescens in relation to temperature, pH, oxygen, carbon dioxide and some nutritional factors. When the temperature was lowered from 30 to 15 C, the minimum a_w increased and the same effect was observed when pH was changed from pH 7.0 to pH 5.4 or pH 8.8.

There are very little data published on a_w limits of Cl. botulinum. Williams and Purnell (1953) reported that growth of Cl. botulinum Type A is inhibited in the range of 0.94 to 0.96 a_w.

Scott (1955) suggested that the minimum a_w for these organisms would be 0.95. He also discussed the growth of these organisms in canned ham and showed that the a_w levels of the samples examined were not low enough to prevent growth.

Kadavy and Dack (1951) conducted a study of the growth of Cl. botulinum in canned bread. They inoculated the bread and found growth in regions where spoilage by other organisms had occurred. In these regions the measured water content was higher, about 9 to 14%, than in the other regions. Water activity was not determined.

Since biologists sometimes consider water availability in terms of the osmotic pressure it is of interest to show the relation between these quantities, thus

$$\text{Osmotic pressure} = \frac{-RT}{V} \ln \frac{p}{p_o}$$

where V is the partial molal volume of water, R is the gas constant, T is the absolute temperature, p_o and p are the vapor pressures of water and the solution respectively. Ingram (1957) showed that the fraction p/p_o represents the "activity" of the water (a_w) for aqueous solutions.

REVIEW OF METHODS FOR DETERMINING ERH

The measurement of the ERH of a substance--that is the relative humidity at which the substance will neither gain nor lose moisture under given temperature conditions--is an important determination that may be used to predict the storage characteristics of food products.

Until recently the ERH of food was not easily measured and food processing practices have been based on the water content of commodities.

The methods used to determine ERH can be classified in the following basic groups:

A. Manometric methods.

B. Electric hygrometer methods.

C. Weight equilibrium methods.

D. Other methods.

Manometric Methods

These methods consist of direct measurements of water-vapor pressure of a closed chamber with a manometer at controlled temperature.

Makover and Myers (1943) described a relatively simple vapor pressure apparatus, consisting of an oil manometer, a sample bottle and a trap.

The apparatus of Vincent and Bristol (1945) is based on the measurement of pressure exerted before and after

freezing of moisture vapor.

Adams and Merz (1929) used an isoteniscope to measure the vapor pressure of saturated salt solutions.

Legault, Makover and Talburt (1948) used a Dubrovin manometer having a sensitivity about seven times that of an ordinary U tube mercury manometer.

Taylor (1961) determined moisture equilibria in dehydrated foods by an improvement of Makover and Myers's (1943) method. The improvement allowed a whole range of observations to be carried out on one sample, whereas the original method required a different sample for each moisture level.

Electric Hygrometer Methods

These methods consist of direct measurement of ERH by the use of an electric hygrometer. An electric hygrometer is an instrument made of two electrodes connected with a hygroscopic salt bridge. As the salt absorbs or loses moisture in equilibrium with the atmosphere to which it is exposed, the electrical resistance of the bridge changes. The resistance can be measured with the appropriate measuring instrument.

Graebner (1944) described a method using an electric hygrometer to measure the relative humidity of the atmosphere after the desired equilibrium, controlled by sulphuric acid, was reached.

Yee (1944) used an electric hygrometer to measure ERH in fertilizers.

Brockington (1949) measured the ERH of whole kernel

corn placing the hygrometer in direct contact with the corn.

Menger (1954) suggested that the ERH in foods might be measured with a hair hygrometer. Generally, however, such methods have a low reliability.

Weight Equilibrium Methods

These methods use the principle that the gain or loss in the moisture content under any given atmospheric relative humidity increases with increasing difference between atmospheric and product vapor pressures. The samples are exposed to controlled atmospheres of different relative humidities and the differences in weights are recorded.

Dittmar (1935) used sulfuric acid of different concentrations to control the relative humidity in humidity chambers.

Makover and Dehority (1943) placed the sulfuric acid of known concentration in vacuum desiccators, which were evacuated to 5 mm of mercury.

Yee and Davis (1944) designed a special humidity chamber where the atmosphere was constantly circulated. Humidity was controlled by supersaturated salt solutions.

Cleland and Fetzer (1944) controlled the humidity by a stream of air of desired relative humidity. The relative humidity of air was adjusted by passing it through saturated salt solutions first and then through sulfuric acid.

Wink's (1947) method differed from the others in that he used a specially designed platform to replace the

left pan of the balance accommodating the crystallizing dishes. This way the samples could be weighed without changing the test conditions.

Hellman and Melving (1948) used a vacuum pump to evacuate the air and reduce the vapor pressure to that provided by saturated salt solutions. After equilibrium was reached the vapor pressure of the atmosphere was measured with a closed-end mercury manometer.

Landrock and Proctor (1951) developed the graphical interpolation method. The method is based on the principle that the rate of gain or loss in the moisture of a product at any given initial moisture content and under any given atmospheric relative humidity increases with increasing difference between atmospheric and product vapor pressures. This method is further discussed under Methods and Materials.

Other Methods

Filter paper strips impregnated with certain salts were used to determine ERH above 90% relative humidity (RH) by Kvaale and Dalhoff (1963). The state--whether dry or wet--of the test papers can be used as an indication of the ERH of a sample when they have been left together in a closed space. This method is further described under Methods and Materials.

When a sensitive and reliable lithium chloride cell for the measurement of the dewpoint of air was announced by Hickes (1947) an attempt was made to adapt this cell for

measuring the ERH of foods. Values for ERH obtained in this way for NaCl solutions correlated satisfactorily with the data derived from measurements according to classical techniques. Determinations with the new method required considerably less time, one hour at the most, versus at least one day in other electric hygrometers as indicated by Waldman and Halvorson (1954) or several days by the older static methods Mossel (1951).

Gur-Arieh, Nelson, Steinberg, and Wei (1965) developed a method to determine the a_w of flour of high moisture content. The flour sample was allowed to equilibrate with water contained in a porous membrane, while under a constant mechanical pressure. After equilibrium was achieved the sample was analyzed for moisture content. A thermodynamic relationship enabled calculation of the a_w corresponding to the pressure used in the cell. Results obtained with this method were in agreement with those obtained with the constant relative humidity desiccator technique in the overlapping range of water activities. The two methods were also in agreement that the a_w of flour was not affected by the particle size distribution.

Monteith and Owen (1958) developed a method using thermo-couples for measuring relative humidity in the range of 95-100%. At high relative humidities a current of 30 milliamperes through a 38 single wire gauge chromel-p/constantan thermocouple produces sufficient Peltier cooling to form a film of water on the junction which may then be used as the wet-bulb of a psychrometer. The method has a

sensitivity of $\pm$ 0.01% relative humidity when measuring the vapor pressure of water in liquid and porous material.

METHODS AND MATERIALS

Methods Used to Determine Equilibrium Relative Humidity

To determine ERH in foods, three different methods were evaluated:

a) The method using humidity sensing elements consisting of lithium chloride sensors and a potentiometer to measure changes in the electrical resistance of the cells due to changes in adsorbed moisture.

b) The salt paper strip method of Kvaale and Dalhoff (1963).

c) The graphical interpolation method of Landrock and Proctor (1951).

Humidity Sensor Method

The first method is a relatively new one which utilizes a Minneapolis-Honeywell W611A relative humidity precision indicator and sensing elements mounted in slotted metal cases which protect the elements without interfering with air circulation over them.

The W611A Portable RH Readout instrument reads relative humidity directly from the atmosphere of almost any area. There are seven different lithium chloride sensors for the different humidity ranges which are accurate over

almost the entire humidity range from 0% to 100%. The instrument is temperature compensated for normal use from 25 to 110 F with special compensation possible below 20 F. This method appeared the most promising of the three. It provides a precise (± 1.0%), but greatly simplified method of determining the relative humidity in most atmospheres where the temperature is between 20 and 140 F, and measurement can be made in a matter of seconds. The portable W611 RH readout instrument is easy to read by using expanded scale plates, each calibrated for a particular sensor range. Each scale plate has three concentric scales: compensation, temperature in degrees F, and RH. The interesting feature of this instrument is its ability to take a reading from a sealed area without the user having to open the vapor seal. The sensor is mounted inside the area and brought through the seal with a vapor-tight connection.

For our purpose, one hundred ml freeze drying flasks were redesigned so that the sensors could be mounted in the upper portion of the flask, leaving the lower portion for the samples. The sensors had their copper leads brought through teflon plugs and were then connected with the meter to read ERH. Each flask could accommodate three sensors or two sensors and a thermometer. The sensors were furnished by the company with ± 1% RH tolerance and with calibration data. Two sensors were obtained in the 80 to 100% and two in the 65 to 90% RH range. To reach equilibrium more quickly a mercury seal glass stirrer was brought through the standard

taper joint normally used to connect the flask to the freeze drying apparatus.

It is essential that the sample, the container, and the sensors be at the same temperature within $\pm$ 0.01 C at all times. A thermostatically controlled bath filled with distilled H_2O adjusted to 30 C was used for this purpose.

Five and ten percent standard sodium chloride (NaCl) solutions giving calculated ERH values of 98.42% and 96.66% were prepared and used to check the sensors. Ten ml were pipetted into the test chamber and with the sensor mounted in the upper part of the flask, the flask was sealed and immersed in the 30 C water bath leaving only the sensor leads and stirrer port exposed. Relative humidity readings were made three times a day. An ERH was reached within 24 to 48 hours. For the first few weeks the sensors seemed to be working properly giving readings close to the calculated values, but the two sensors were in disagreement with one always sensing lower RH values than the other. Also, relatively minor changes in the water level of the bath lowered the RH value considerably, which could not be explained by temperature difference alone. The air temperature above the water was the same as the water temperature since the bath was covered.

When an incubator with constant temperature was used to replace the water bath, fairly constant RH values were obtained but they were much lower than the calculated values for the NaCl solutions used.

The experimentally determined RH values presented in Table 1 were obtained when the actual RH was 96.66%. The table also revealed that one of the sensors was registering 2% less RH than the other. This should not be the case since the calibration curve provided by the manufacturer showed very good agreement between these two sensors.

The low readings obtained could be an indication that the sensors require recalibration since with time the calibration of these devices change and for very precise work they may have to be returned to the manufacturer for recalibration, possibly after each experiment.

Table 1. Percent ERH obtained with sensors using 10% NaCl as medium.

	Time in hours			
Sensor No.	0	24	48	72
246-2	88.3	89.8	90.2	89.8
246-41	90.2	91.2	91.5	91.3

Salt-paper Strip Method

To ensure that the sensors were registering lower RH values in the chambers than the actual percent RH, we turned to our second method, the salt-paper strip method. This method uses strips of filter paper impregnated with certain salts. The state (whether dry or wet) of such test papers can be used as a direct indication of the ERH of a sample when the papers and the sample have been left together for some time in a closed space. This method was developed

primarily for the measurement of humidities above 90% RH for use in studies of prepacked meat products.

Filter paper strips were impregnated with salts covering the range of 90 to 100% RH and were placed in the test containers together with the sensors and 5 ml of 10% NaCl was added to control a_w. An ERH was reached usually in 48 to 72 hours. Salt strips showed RH values in agreement with the calculated ones; 96 to 97% RH for 10% NaCl, while one of the sensors registered 87.8% and the other one registered 86.4% RH.

Salt paper strips were also tested in plastic petri dishes and the results obtained were in good agreement with those obtained previously when the strips were placed together with the sensors. A 97.7 to 100% RH was obtained for a 5% NaCl solution (calculated percent RH is 98.42). The salt paper strip method was less sensitive to temperature changes than were the sensors. They could be placed at room temperature without any difficulties caused by minor temperature changes. Smaller containers are more advantageous since the smaller the air space, the sooner an ERH is reached.

It is well known that ERH determinations are rather uncertain at high humidity levels. Some of the salts gave uncertain results. In some instances, the strips got wet when the percent RH indicated by the other salts was lower than indicated by the first salt. Most of the uncertainties occurred above 96% RH which is the range in which we are

most interested. This discrepancy in the salt paper method and the limited accuracy with the first method focused attention to a third method; namely, the "graphical interpolation method."

Graphical Interpolation Method

This method requires that samples of the product under test be brought to weight equilibrium when exposed to a series of different RH. It is generally used for food products, gives satisfactory results with simple equipment, but requires numerous weighings and is time-consuming.

Five ml of liquid media or 5 g of solid media were put into petri dishes and weighed to the nearest 0.1 mg and then placed in a Stender dish containing various sulfuric acid solutions to produce atmospheres of known RHs. The samples were exposed to these controlled atmospheres for 48 hours at room temperature. After the exposure period, the gain or loss in weight of each of the samples was determined (Table 2). These values were then plotted with reference to the RHs with the gains in weight above and the losses in weight below a horizontally drawn "zero baseline" representing no change in weight. Interpolation of the ERH was made at that point where the smooth curve intersected the zero baseline (Figure 1).

To check the accuracy of the method, 4.0, 4.5, 5.0 and 10.0% (w/w) NaCl solutions were used as standards having calculated ERH values of 98.95, 98.62, 98.48 and 96.91%,

Table 2. Graphical interpolation data for TPSY medium with KCl to control a_w.

TPSY with % KCl (w/v)	Change (mg) in weight per hour at relative humidity (%) of					
	96.8	97.4	97.8	98.2	98.7	99.3
4.5	-	-1.00	-0.30	+0.46	+1.55	+2.88
	-	-1.30	-0.38	+0.50	+1.34	+2.58
5.0	-	-0.66	+0.06	+0.82	-	-
	-	-0.72	+0.19	+0.92	-	-
5.5	-1.10	-0.24	+0.30	-	-	-
	-1.60	-0.60	+0.38	-	-	-
6.0	-1.01	-0.11	+0.90	-	-	-
	-0.70	-	+0.94	-	-	-
6.5	-1.07	0.00	-	-	-	-
	-0.94	+0.07	+1.01	-	-	-

respectively. The ERH values obtained with the method are tabulated in Table 3 and show very good agreement with the calculated values and were readily reproducible. Since this method exhibited the best accuracy, it was thus the method of choice for our further studies.

Microorganisms: The microorganisms studied in the experiments were four strains of Cl. botulinum type E. The strains and their sources were:

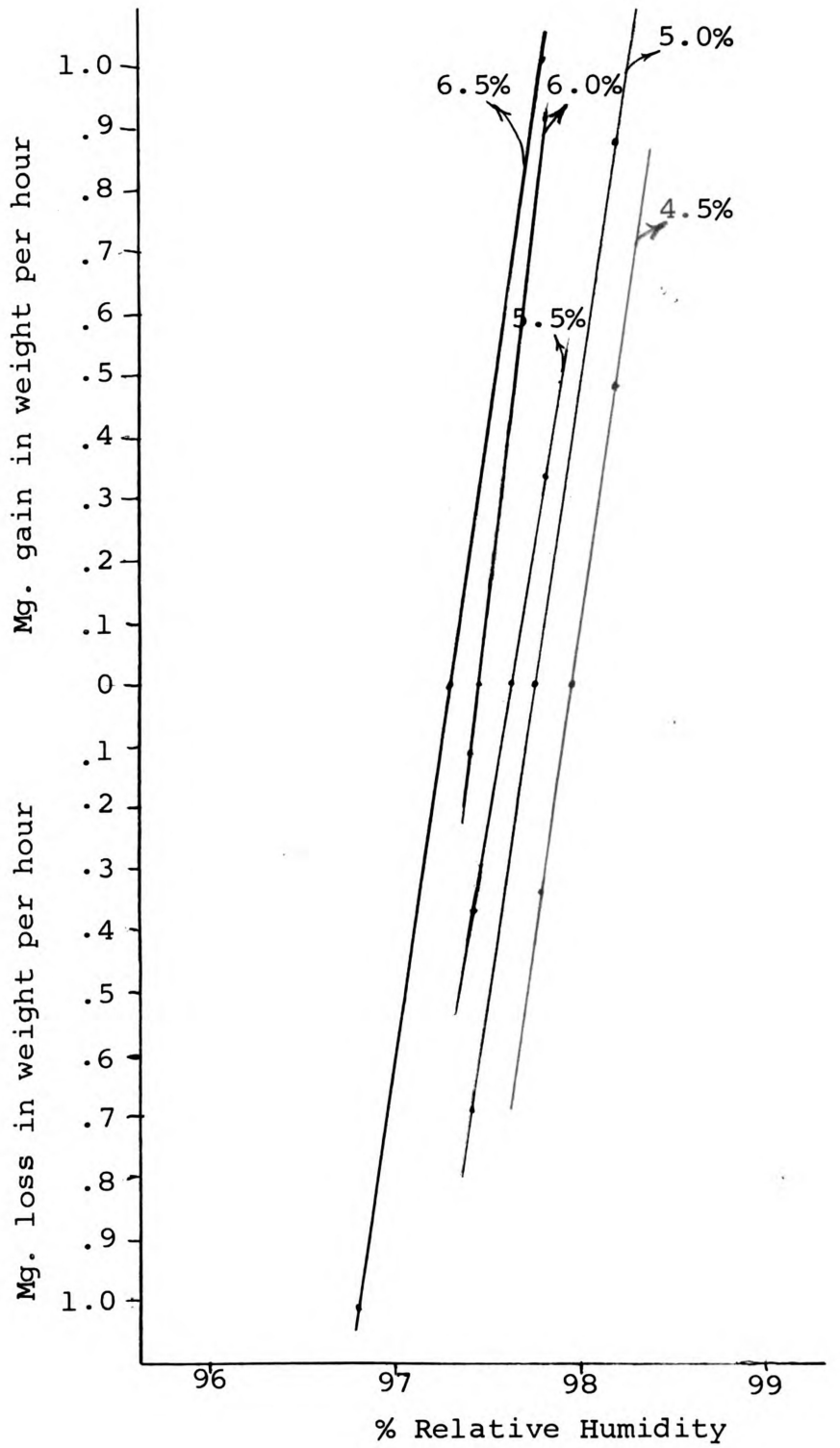

Figure 1. Graphical interpolation isotherm for TPSY medium with KCl to control water activity.

Table 3. Percent ERH values of NaCl standards.

Percent NaCl Std. (w/w)	Percent RH determined	Percent RH calculated
4.0	98.99	98.95
4.5	98.91	98.62
5.0	98.82	98.48
10.0	95.42	96.91

Name of strain	Source
Kalamazoo (K)	Mr. R. W. Johnston, Food and Drug Administration, Detroit, Mich.
Vancouver Herring (VH)	Dr. C. F. Schmidt, Continental Can Co., Chicago, Ill.
Seattle Forks (SF)	Dr. J. T. Graikowski, Bureau of Commercial Fisheries, Ann Arbor, Mich.
A 6247	Mr. R. W. Johnston, Food and Drug Administration, Detroit, Mich.

Viable count procedure: Viable counts of spore suspensions were obtained using Beef Infusion medium of the following formula:

Beef infusion	1 liter
Bacto peptone	1%
Disodium phosphate	0.2%
Glucose	0.2%
Sodium chloride	0.5%
Sodium thioglycollate	0.1%
pH 7.2	
Agar	1.5%

Appropriate dilutions were prepared so that aliquots of the dilutions would produce 10 to 50 colonies per 16 x 150 mm screw cap test tube. Colonies were counted after 24 to 36 hours incubation at 30 C using triplicate tubes per dilution.

Toxicity assays: Toxicity was tested using intraperitoneal injections into 15-20 g Swiss Webster white mice. One ml of a sample was digested with 1 ml of 1% trypsin solution for 60 minutes at 37 C. A volume of 0.2 ml of this digest was injected into a mouse. A second mouse used as control also received 0.2 ml of digest but was protected with 0.1 ml of type E antitoxin (1:200 dilution of the Porton antitoxin.) Death of the unprotected mouse within 96 hours was considered a positive test.

Solute-containing Media: All the water activity and osmotic pressure studies were conducted in Trypticase Peptone Sucrose Yeast extract broth medium (TPSY) to which various solutes were added.

The TPSY formula used was:

Trypticase	5%
Peptone	0.5%
Yeast extract	1.0%
Sucrose	0.2%
Na thioglycollate	0.02%
pH 7.2	

RESULTS AND DISCUSSION

In our previous unpublished studies, it was established that 4.87% NaCl in TPSY medium inhibited the growth of Cl. botulinum Type E strains at 60 and 50 F incubation temperatures. At lower temperatures such as 45 F, 4.17 to 4.6% was the inhibitory NaCl range and at 41 F, 2.5 to 3.0% were inhibitory depending on the strains.

To determine if the inhibition of outgrowth was a result of lowered a_w of the media due to the added NaCl, ERH relationships were examined at various temperatures using NaCl and other solutes.

Water Activity

The ERH values of 4.0, 4.5, 5.0 and 10.0% NaCl solutions were determined and are presented in Table 3. After satisfactory results were obtained with the standard NaCl solutions, the ERH of TPSY media containing 2.0-5.0% NaCl were determined (Table 4). The ERH values obtained with the TPSY plus NaCl media are somewhat lower than those obtained for pure NaCl solutions, but this is quite understandable since this medium is composed of several different ingredients, all of which would tend to depress the ERH.

From the data in Table 4, it seems that the inhibitory value of ERH for Type E Cl. botulinum in TPSY media with NaCl

Table 4. Equilibrium relative humidity values of TPSY media containing various amounts of NaCl.

Percent NaCl (w/v)	ERH
5.0	97.54
4.5	97.83
4.0	98.05
3.5	98.34
3.0	98.50
2.5	98.61
2.0	98.90

lies between 97.83 and 97.54%.

Additional salts such as sodium formate (M.W. 68.01) and KCl (M.W. 74.55) were used to determine if this inhibition was due to a small amount of available water in the media or if NaCl was acting in higher concentration solely as a chemical preservative, or was an ionic effect due to high concentration of certain ions or an ion imbalance. Some nonelectrolytes such as sucrose, galactose, and glycerol were also evaluated.

TPSY media with these electrolytes and non-electrolytes were prepared and concentrations were adjusted so that the inhibitory range previously obtained with NaCl would be covered. The ERH of these media containing the different concentrations of salts and carbohydrates was then determined (Table 5). The obtained ERH data agreed very well with the calculated data minus the ERH value of the TPSY medium.

The ERH determinations showed that 35% sucrose decreased the a_w of the TPSY medium to 0.9766 and 12.5% glycerol decreased the a_w to 0.9648, while 5.5% KCl reduced the a_w of the medium to 0.9762 and 5.0% sodium formate decreased it to 0.9761. According to the NaCl data these a_w values may not permit growth. To investigate this, the different concentrations of these solutions were inoculated with Type E strains of Cl. botulinum. Four strains; K, A6247, SF, and VH were used for the sucrose studies, while the SF and VH strains were used for the KCl test and the VH

Table 5. Percent ERH values of TPSY medium with NaCl, KCl, NaCOOH, Sucrose and Glycerol.

Percent Solute (w/v)	Percent ERH	a_w
NaCl		
2.0	98.90	0.9890
2.5	98.61	0.9861
3.0	98.50	0.9850
3.5	98.34	0.9834
4.0	98.05	0.9805
4.5	97.83	0.9783
5.0	97.54	0.9754
KCl		
4.5	97.97	0.9797
5.0	97.76	0.9776
5.5	97.62	0.9762
6.0	97.44	0.9744
6.5	97.31	0.9731
NaCOOH		
3.5	98.50	0.9850
4.0	98.03	0.9803
4.5	97.91	0.9791
5.0	97.61	0.9761
5.5	97.08	0.9708
6.0	96.94	0.9694
6.5	96.41	0.9641
Sucrose		
18.5	99.03	0.9903
22.8	98.76	0.9876
26.7	98.50	0.9850
31.0	97.97	0.9797
35.0	97.66	0.9766
38.5	97.55	0.9755
Glycerol		
2.5	99.20	0.9920
5.0	98.57	0.9857
7.5	98.13	0.9813
10.0	97.83	0.9783
12.5	96.48	0.9648
15.0	95.40	0.9540

and K strains were used for the studies with sodium formate. Three screw cap test tubes of each concentration containing 15 ml of solution were inoculated with suspensions containing 0.1 ml of 1.5×10^7 spores/ml. The tubes were then sealed with Vaspar (mixture of 25% wax and 75% petroleum jelly) and incubated at 30, 21.2, 15.6, 10, and 7.2 C. Growth was observed by turbidity and gas production and toxicity was checked by mouse toxin assay. Only those tubes were considered having type E outgrowth which showed gas and gave a positive toxicity test.

Experiments using galactose could not be carried out since the amount required to depress the ERH to the 97.66% value was above the level of solubility of this carbohydrate.

Sucrose was very soluble in the TPSY medium and dissolved easily up to 38.5% concentration (w/v).

The four strains tested produced good growth at all five temperatures in the triplicate tubes of TPSY medium with 31% sucrose concentration at a_w 0.9797 (Table 6). However, none of them was able to grow in 35% sucrose concentration at a_w 0.9766, except that in one instance one tube inoculated with the SF strain showed outgrowth at the 21.1 C incubation temperature after 29 days. This could be an indication of strain variation, in that this strain was capable of growth at a somewhat lower a_w than the other strains. The tubes inoculated with the K, A6247 and SF strains showed growth after one or two days incubation in the 31% sucrose concentration at 30 C, while the VH strain

Table 6. Outgrowth time in days of four Type E Cl. botulinum strains incubated 130 days in TPSY medium with sucrose used to control a_w.

Sucrose conc. % (w/v)	a_w	Strain	Incubation temperature (C)				
			30	21.1	15.6	10	7.2
		K	*	*	*	*	10,19,19
18.5	0.9903	A6247	*	*	*	*	21,21,21
		VH	*	*	*	*	40,40,40
		SF	*	*	*	*	21,21,21
22.8	0.9876	K	*	*	*	19,19,19	19,19,19
		A6247	*	*	*	21,21,21	21,21,21
		VH	*	*	*	- - -	45,45,45
		SF	*	*	*	21,21,21	21,21,21
26.7	0.9850	K	1,1,1	2,2,2	4,4,4	27,27,27	35,35,35
		A6247	2,2,2	2,2,2	6,6,6	21,21,21	29,29,29
		VH	1,1,1	4,4,4	6,6,6	55,55,55	- - -
		SF	2,2,2	2,2,2	3,3,3	21,21,21	37,37,37
31.0	0.9797	K	1,4,4	5,5,5	5,5,6	35,35,35	39,39,39
		A6247	2,2,2	3,3,7	7,7,7	37,37,37	39,39,39
		VH	4,4,4	12,12,12	15,18,20	67,67,67	86,86,86
		SF	2,2,2	3,3,3	7,7,7	29,29,29	39,39,39
35.0	0.9766	K	-	-	-	-	-
		A6247	-	-	-	-	-
		VH	-	-	-	-	-
		SF	-	29 - -	-	-	-
38.5	0.9755	K	-	-	-	-	-
		A6247	-	-	-	-	-
		VH	-	-	-	-	-
		SF	-	-	-	-	-

* No sample was used.
- No growth was observed.

required a lag period of 4 days. At lower temperatures this difference in the lag time became more pronounced. At 21.1 C, the lag time of the VH strain increased to 12 days, at 15.6 C to 15 to 20 days, and at 10 C to 67 days. The comparable lag times of the other three strains were 5 to 7, 5 to 7 and 29 to 35 days, respectively.

Microscopic observation of the cultures in sucrose solution showed normal cell structure with some chain formation when incubated at 15.6 C or higher. Sporulation was also readily observed.

Control of a_w by glycerol did not produce the expected results. A concentration of 12.5% glycerol (a_w 0.9648) increased the lag time but did not inhibit growth, although at this a_w level an inhibition of growth was observed with the other compounds. Even when the glycerol concentration of the medium was increased to 15% (a_w 0.9540) there was no inhibition of growth at 30 and 21.1 C but an increased lag time occurred. The ERH values determined for these two glycerol concentrations were much lower than found by calculation (12.5% measured ERH = 96.48%, calculated = 97.60%; 15% measured ERH = 95.40%, calculated = 97.14%). Why glycerol, the alcohol form of a three carbon sugar, does not inhibit growth at the same a_w ranges as the other investigated compounds is not as yet known. There is no clear cut answer to this problem yet, and until further evidence is obtained we have to settle with a negative answer.

When the a_w was reduced with KCl (Table 7), good

Table 7. Outgrowth time in days of SF and VH Type E Cl. botulinum strains incubated 130 days in TPSY medium with KCl used to control a_w.

KCl conc. % (w/v)	a_w	Strain	Incubation temperature (C)				
			30	21.1	15.6	10	7.2
4.5	0.9797	SF	2,2,6	6,6,6	6,6,6	-	-
		VH	5,5,5	5,5,5	8,8,8	-	-
5.0	0.9776	SF	6 - -	6,6,6	6,6,6	-	-
		VH	5,5,8	11,11,32	14,14,22	-	-
5.5	0.9762	SF	-	-	10,20,+	-	-
		VH	18 -	-	-	-	-
6.0	0.9744	SF	-	-	-	-	*
		VH	-	-	-	-	*
6.5	0.9731	SF	-	-	-	*	*
		VH	-	-	-	*	*

growth was observed up to 5.0% KCl concentration (a_w 0.9776), while only some sporadic growth was observed at the 5.5% (a_w 0.9762), concentration. One tube inoculated with the VH strain showed growth after 18 days at 30 C, and two tubes inoculated with the SF strain produced growth after 10 and 20 days at 15.6 C.

In the medium where a_w was controlled with sodium formate (Table 8), outgrowth occurred at the 5.0% level (a_w 0.9761), and at lower concentrations, but not at the 5.5% concentration (a_w 0.9708).

Microscopic observation of the cultures grown in the KCl and sodium formate controlled media showed filamentous structure of the vegetative cell and long chains. Sporulation was never observed.

The inhibition of growth with the tested solutions could be inhibition due to a_w. Since no growth has been observed with concentrations of 38.5% sucrose (a_w 0.9755), 6.0% KCl (a_w 0.9744), 5.5% sodium formate (a_w 0.9708), and 5.0% NaCl (a_w 0.9754) thus far, the inhibitory a_w level of growth for the tested Type E strains in these solutions appears to be between 0.9708 and 0.9755, if inhibition is due to a_w.

The slight difference between the expressed inhibition by the salts and the sucrose may be an indication of a difference in ionic concentration or it could be merely an experimental error. Thus, the possibility of ionic effects were examined further.

Ionic Effect: Sucrose in the media inhibited outgrowth at the 0.9755 a_w level while NaCl, Na formate, KCl

Table 8. Outgrowth time in days of K and VH Type E Cl. botulinum strains incubated 130 days in TPSY medium with Na formate used to control a_w.

Na formate conc. % (w/v)	a_w	Strain	Incubation temperature (C)				
			30	21.1	15.6	10	7.2
3.5	0.9850	K	*	*	*	*	30,30,30
		VH					30,30,30
4.0	0.9803	K	*	*	*	-	30 - -
		VH				42,42,42	30,42,42
4.5	0.9791	K	3,3,3	14,14,14	14,14,14	-	-
		VH	3,3,4	4,4,4	14,14,14	-	42,53 -
5.0	0.9761	K	4,14,22	14,14,22	22 - -	-	-
		VH	4,4, -	14,14,14	14,22 -	-	-
5.5	0.9708	K	-	-	-	-	-
		VH	-	-	-	-	-
6.0	0.9694	K	-	-	-	-	*
		VH	-	-	-	-	*
6.5	0.9641	K	-	-	-	*	*
		VH	-	-	-	*	*

inhibited at a_w levels 0.9744 to 0.9708. The difference in a_w values was not appreciable. Although the concentration of ions was high in the electrolyte solutions and practically no ions were present in the non-electrolyte solution, the a_w levels inhibiting growth were very close.

The following calculations show the percentage composition of the salts:

NaCl (M.W. 58.45)	NaCOOH (M.W. 68.01)	KCl (M.W. 74.55)
Na = 39.2%	Na 33.6%	K 52.5%
Cl 60.6%	C 17.6%	Cl 47.5%
	O_2 46.9%	
	H 1.4%	

The above calculations reveal that the percentage of sodium is different in the two sodium salts. NaCl contains 6.4% more Na ion than sodium formate. The KCl salt contains 52.5% K ion. The anions are also in a different ratio, 60.0% Cl ion in NaCl and 47.5% in KCl, while in sodium formate there is 65.9% formate; however, all three salts inhibited outgrowth within close a_w levels, and at the same approximate a_w levels the non-electrolyte sucrose was inhibitory.

The lag times at 30 C for the VH and K strains in 4.5% sodium formate concentration (a_w 0.9791) were 3 to 4 days, 2 to 6 days in 4.5% KCl for the SF strain and was 5 days for the VH strain. At the same incubation temperature in 35% sucrose (a_w 0.9797), the SF and A6247 strains required only 2 days and the VH and K strains required 4 days

to show growth. At almost identical a_w levels the ability of the strains to germinate and start to grow was only slightly different. They all started to grow in 2 to 3 days except the VH strain. This strain seems to require somewhat more time to show growth in the sucrose medium and even more time at lower temperatures. Also, the minor differences existing among the other strains could indicate either a strain difference or an ionic effect.

The possibility of osmotic effect was also investigated. Table 9 shows that the 38.5 sucrose solution, which is approximately 1.12 molar (M) has an osmotic pressure of about 25.08 atmospheres. A 6.0% solution of KCl is 0.80 M with respect to both K^+ and Cl^- ions, hence its osmotic pressure is equal 2 x 0.80 x 22.4 or about 36.0 atmospheres. A 5.5% sodium formate solution which is 0.80 M produces an osmotic pressure of about 35.8 atmospheres, while a 5.0% NaCl solution is 0.86 M and produces an osmotic pressure of about 38.5 atmospheres.

The osmotic pressure of a 15%, 1.63 M glycerol solution is about 36.5 atmospheres and at this molarity or osmotic pressure, glycerol still permitted outgrowth.

The molar concentrations of the inhibiting salt concentrations are similar (0.80 to 0.86) and thus so are the osmotic pressures. The molar concentration and the calculated osmotic pressure of the inhibiting sucrose solution (1.12 M) is different from that of the salts. Therefore, the growth inhibiting characteristics of the solutes tested thus

Table 9. Calculated molar concentration and osmotic pressure values of glycerol, sucrose, NaCl, KCl and sodium formate solutions at 0 C.

Percent solute (w/v)	Molarity	Osmotic pressure in atmospheres
Glycerol		
10.0	1.08	24.20
15.0	1.63	36.50
20.0	2.17	48.61
Sucrose		
10.0	0.29	6.54
22.8	0.66	14.78
26.7	0.78	17.47
31.0	0.90	20.16
35.0	1.02	22.84
38.5	1.12	25.08
NaCl		
4.5	0.77	34.40
4.7	0.81	36.28
5.0	0.86	38.52
10.0	1.72	77.04
KCl		
4.5	0.60	27.01
5.0	0.67	30.02
5.5	0.73	33.00
6.0	0.80	36.02
Sodium Formate		
4.0	0.58	25.98
4.5	0.66	29.56
5.0	0.73	32.70
5.5	0.80	35.80

might not be explained using the molar concentrations of solutes that were added to the TPSY medium.

However, the gas law equation is valid only at low solute concentrations and osmotic pressure generally increases with higher sucrose concentrations much more rapidly than predicted by the simple equation used to calculate osmotic pressure. The inhibiting sucrose concentration could have an osmotic pressure similar to those calculated for the salts. Therefore, the growth inhibiting characteristics of the solutes tested thus may also be explained with the osmotic phenomenon, if selective permeability is involved. The glycerol data are not readily explained by either the a_w theory or osmotic pressure phenomenon and should be the subject of further study.

Microscopic examination of the cultures showed that with sucrose in the medium the bacteria grew normally and only an increase of lag time was noticed. Also the rate of growth was not affected and sporulation followed growth. In the salt-controlled medium the cultures showed long filamentous vegetative cells, chains, some curved rods, and a few straight rods. Sporulation was never observed. These differences in the electrolyte and non-electrolyte controlled media indicate that the salts beside lowering the a_w may also affect the normal growth of the bacteria.

Temperature effect: Generally temperature had an effect on the lag period, the rate of growth, and the number of cells produced. The lag time increased with lower

incubation temperatures, and the rate of growth and the amount of cellular material produced decreased. The optical density (OD) of the cultures dropped from an absorbancy of 0.7 to 0.1 at 625 mμ as the temperature decreased from 30 to 7.2 C.

When 31% sucrose concentration (a_w 0.9766), was used to control the a_w at 30 C all four strains started to grow within 1 to 4 days, while at 21.1 C the lag times were slightly increased and with the exception of the VH strain they showed growth in 3 to 7 days. The VH strain required 12 days to initiate growth. At 15.6 C, the lag times were again increased a few days, while at 10 C the increase was very substantial. The K, SF, and A6247 strains started to grow only after 21 to 37 days incubation. The VH strain had the longest lag time of 67 days.

With less sucrose in the medium, the a_w level is increased and the microorganisms can better tolerate the adverse effect of lower incubation temperatures which results in reduced lag times, increased growth rates, and an increased production of cells. This is well demonstrated with the 26.7% sucrose concentration in the medium. Four percent less sucrose in the medium increased the a_w from 0.9797 to 0.9850, and at 10 C the lag time was decreased by 8 days for the K strain, 16 days for the A6247 strain, 12 days for the VH strain, and 8 days for the SF strain.

In the media containing KCl and sodium formate, the observed lag times at 30, 21.1 and 15.6 C incubation temperatures were very similar to those obtained from the experiment

with sucrose. However, at the lower temperatures (10 and 7.2 C) no growth was observed with the SF and VH strains in the 4.5% (a_w 0.9797), and 5.0% (a_w 0.9776) KCl media, while the 4.5% (a_w 0.9791) sodium formate concentration inhibited the outgrowth of the K strain but not the VH strain. This strain showed growth after 42 and 53 days incubation at 7.2 C. If these findings are compared with the data obtained with the sucrose controlled media the data show that at a_w 0.9797 all four strains grew after a lengthy incubation time. This may indicate that at low temperatures such as 10 and 7.2 C the electrolytes are a little more effective in inhibiting growth, or that the actual a_w is slightly different from the recorded one.

The inhibitory a_w levels of the K, A6247, VH and SF strains were measured as 0.9755, 0.9708 and 0.9744 when sucrose, sodium formate, and KCl, respectively, were used to control the a_w of the TPSY medium. Strain variation may produce growth at slightly lower a_w levels, but since no growth has been observed at a_w 0.9755 (sucrose), a_w 0.9708 (sodium formate) and a_w 0.9744 (KCl) thus far, the inhibitory level of growth for the investigated Type E strains in these solutions appears to be between the 0.9708 and 0.9755 a_w levels, if inhibition is due to a_w.

The Effect of Preheating and the Size of the Inoculum on Outgrowth in Water Activity Media

The a_w range of the K, A6247, VH, and SF strains was established in the previous experiment, using a 1.0×10^6

spores/ml inoculum. To determine how the size of inoculum and preheating spores before inoculation affects outgrowth the following sizes of inocula were used; 1 x 10^6/ml, 1 x 10^4/ml and 1 x 10^2/ml. All three inocula were tested with and without heat shock. The spore suspensions were heat treated for 15 minutes at 60 C. Since, at this temperature a number of spores would be injured or killed, the suspensions were readjusted based upon viable counts to compensate for this loss. Spores were inoculated and incubated in the a_w media at five different temperatures as in the previous experiment, but only the K and VH strains were used in this experiment.

Effect of the size of the inoculum: In the sucrose medium (Tables 10, 10a) both strains produced outgrowth in the 31% concentration (a_w 0.9797) but not in the 35% concentration (a_w 0.9766). The size of the inoculum affected lag times, but not outgrowth of the VH strain. The lag time increased significantly as the size of the inoculum decreased in the 15.6-21.1 C temperature range. At higher or lower temperatures only the K strains showed appreciable differences in the lag times due to inoculum size. This strain showed poor outgrowth even at 30 C when the 1.0 x 10^2/ml inoculum was used, and at the 7.2 to 10 C temperature range no outgrowth at all was observed. The VH strain showed outgrowth at 30 C in 2 to 3 days in all tubes regardless of the inoculum size, while in the 7.2 to 10 C temperature range outgrowth was observed only after 32 days of incubation.

Table 10. The effect of inoculum size and preheating upon outgrowth time in days of the VH Type E Cl. botulinum strain incubated 90 days in TPSY medium with sucrose to control a_w.

Sucrose conc. % (w/v)	a_w	Size of inoculum	Incubation temperature (C)				
			30	21.1	15.6	10	7.2
31.0	0.9797	1.0×10^6H	2,2,2	6,6,6	12,12,12	20,20,20	33,33,33
		1.0×10^6	2,2,2	3,3,7	7,7,7	37,37,37	35,35,35
		1.0×10^4H	2,3,3	11,11,32	15,19,46	32,32,32	32,46,-
		1.0×10^4	2,2,2	11,11,11	15,15,15	32,32,32	32,32,32
		1.0×10^2H	3,3,3	11,13,-	21,21,-	46,46,-	32,- -
		1.0×10^2	2,2,-	10,10,10	24,24,24	32,32,32	32,32,32
35.0	0.9766	1.0×10^6H	-	-	-	-	-
		1.0×10^6	-	-	-	-	-
		1.0×10^4H	-	-	-	-	-
		1.0×10^4	-	-	-	-	-
		1.0×10^2H	-	-	-	-	-
		1.0×10^2	-	-	-	-	-
38.5	0.9755	1.0×10^6H	-	-	-	-	-
		1.0×10^6	-	-	-	-	-
		1.0×10^4H	-	-	-	-	-
		1.0×10^4	-	-	-	-	-
		1.0×10^2H	-	-	-	-	-
		1.0×10^2	-	-	-	-	-

- No growth was observed.
H-Spore suspension was heat treated.

Table 10a. The effect of inoculum size and preheating upon outgrowth time in days of the K Type E Cl. botulinum strain incubated 90 days in TPSY medium with sucrose to control a_w.

Sucrose conc. % (w/v)	a_w	Size of inoculum	Incubation temperature (C)				
			30	21.1	15.6	10	7.2
31.0	0.9797	1.0×10^6 H	3,3,3	14,14,-	20,41,-	41,41,41	41,41,56
		1.0×10^6	1,4,4	5,5,5	5,5,6	35,35,35	39,39,39
		1.0×10^4 H	6,8,-	27,41,-	27,27,27	41,- -	41,- -
		1.0×10^4	5,5,5	19,19,19	26,26,26	40,40,55	- - -
		1.0×10^2 H	- - -	- - -	28,- -	- - -	- - -
		1.0×10^2	13, -	26,- ,26	40,40,-	- - -	- - -
35.0	0.9766	1.0×10^6 H	- - -	- - -	- - -	- - -	- - -
		1.0×10^4 H	- - -	- - -	- - -	- - -	- - -
		1.0×10^4	- - -	- - -	- - -	- - -	- - -
		1.0×10^2 H	- - -	- - -	- - -	- - -	- - -
		1.0×10^2	- - -	- - -	- - -	- - -	- - -
38.5	0.9755	1.0×10^6 H	- - -	- - -	- - -	- - -	- - -
		1.0×10^4 H	- - -	- - -	- - -	- - -	- - -
		1.0×10^4	- - -	- - -	- - -	- - -	- - -
		1.0×10^2 H	- - -	- - -	- - -	- - -	- - -
		1.0×10^2	- - -	- - -	- - -	- - -	- - -

When sodium formate (Tables 11, 11a) was used to control a_w, outgrowth was observed in the 4.5% concentration (a_w 0.9791), while in the 5% concentration (a_w 0.9761) only the 1.0×10^6 spores/ml inoculum produced outgrowth in the 15.6 to 30 C temperature range. Again, in this medium the outgrowth of the K strain was affected more than the outgrowth of the VH strain in the lower (1.0×10^2/ml and 1.0×10^4/ml) inoculum ranges. These inocula were inhibited at the lower (7.2 and 10 C) incubation temperatures. The K strain exhibited poor outgrowth at the higher temperatures as well. Only two tubes out of the three were positive with the 1.0×10^2 spores/ml inoculum at 21.1 C and no outgrowth was observed at 15.6 C.

In the KCl medium (Tables 12, 12a) the two strains showed widely different outgrowth patterns. The VH strain produced outgrowth in the 5.5% concentration (a_w 0.9762) while the K strain was completely inhibited at this concentration. Both strains were inhibited in the 7.2 to 10 C temperature range in the 4.5% KCl concentration (a_w 0.9797). The 1.0×10^2 spores/ml dilution of the K strain was inhibited even in the 4.5% concentration (a_w 0.9797) except for one tube, which was positive after 10 days of incubation at 30 C. The VH strain showed erratic response in the higher dilutions.

The effect of preheating: No beneficial effect of the heat shock was observed with the K strain. The VH strain produced outgrowth in 4.5% (a_w 0.9791) sodium formate media (Table 11), when heat shocked and incubated at 10 and

Table 11. The effect of inoculum size and preheating upon outgrowth time in days of the VH Type E Cl. botulinum strain incubated 90 days in TPSY medium with sodium formate to control a_w.

Sodium formate conc. % (w/v)	a_w	Size of inoculum	Incubation temperature (C)				
			30	21.1	15.6	10	7.2
4.5	0.9791	1.0×10^6H	2,2,3	6,6,8	9,9,9	26,26,26	33,- ,-
		1.0×10^6	3,3,4	4,4,4	14,14,14	- - -	30,- ,-
		1.0×10^4H	3,3,4	8,11,14	11,13,13	32,32,32	32,32,46
		1.0×10^4	3,3,3	9,9,11	11,11,14	- - -	- - -
		1.0×10^2H	3,3,5	11,11,11	11,11,11	- - -	- - -
		1.0×10^2	6,6,10	14,14,45	12,14,18	- - -	- - -
5.0	0.9761	1.0×10^6H	- - -	- - -	- - -	- - -	- - -
		1.0×10^6	4,4,-	14,14,14	14,22,-	- - -	- - -
		1.0×10^4H	- - -	- - -	- - -	- - -	- - -
		1.0×10^4	- - -	- - -	- - -	- - -	- - -
		1.0×10^2H	- - -	- - -	- - -	- - -	- - -
		1.0×10^2	- - -	- - -	- - -	- - -	- - -
5.5	0.9708	1.0×10^6H	- - -	- - -	- - -	- - -	- - -
		1.0×10^6	- - -	- - -	- - -	- - -	- - -
		1.0×10^4H	- - -	- - -	- - -	- - -	- - -
		1.0×10^4	- - -	- - -	- - -	- - -	- - -
		1.0×10^2H	- - -	- - -	- - -	- - -	- - -
		1.0×10^2	- - -	- - -	- - -	- - -	- - -

Table 11a. The effect of inoculum size and preheating upon outgrowth time in days of the K Type E Cl. botulinum strain incubated 90 days in TPSY medium with sodium formate to control a_w.

Sodium formate conc. % (w/v)	a_w	Size of inoculum	Incubation temperature (C)				
			30	21.1	15.1	10	7.2
4.5	0.9791	1.0×10^6H	3,3,6	10,10,14	20,20,27	- - -	- - -
		1.0×10^6	3,3,4	4,4,4	14,14,14	- - -	42,53,-
		1.0×10^4H	6,6,8	10,10,-	- ,33,33	- - -	- - -
		1.0×10^4	5,7,7	13,13,13	25,25,25	- - -	- - -
		1.0×10^2H	- - -	20,- -	- - -	- - -	- - -
		1.0×10^2	5,5,5	19,26,-	- - -	- - -	- - -
5.0	0.9761	1.0×10^6H	- - -	- - -	- - -	- - -	- - -
		1.0×10^6	4,14,22	14,14,22	42,- -	- - -	- - -
		1.0×10^4H	- - -	- - -	- - -	- - -	- - -
		1.0×10^4	- - -	- - -	- - -	- - -	- - -
		1.0×10^2H	- - -	- - -	- - -	- - -	- - -
		1.0×10^2	- - -	- - -	- - -	- - -	- - -
5.5	0.9708	1.0×10^6H	- - -	- - -	- - -	- - -	- - -
		1.0×10^6	- - -	- - -	- - -	- - -	- - -
		1.0×10^4H	- - -	- - -	- - -	- - -	- - -
		1.0×10^4	- - -	- - -	- - -	- - -	- - -
		1.0×10^2H	- - -	- - -	- - -	- - -	- - -
		1.0×10^2	- - -	- - -	- - -	- - -	- - -

Table 12. The effect of inoculum size and preheating upon outgrowth time in days of the VH Type E Cl. botulinum strain incubated 90 days in TPSY medium with KCl to control a_w.

KCl conc. % (w/v)	a_w	Size of inoculum	Incubation temperature (C)				
			30	21.1	15.6	10	7.2
4.5	0.9797	1.0×10^6H	2,4,4	4,8,12	20,20,20	- - -	- - -
		1.0×10^6	5,5,5	5,5,5	8,8,8	- - -	- - -
		1.0×10^4H	3,3,3	11,13,13	19,19,25	- - -	- - -
		1.0×10^4	2,3,2	7,7,25	15,15,15	- - -	- - -
		1.0×10^2H	- - -	- - -	32,- -	- - -	- - -
		1.0×10^2	31, -	12,14,24	18,- -	- - -	- - -
5.0	0.9776	1.0×10^6H	6,6,6	12,16,16	26,26,26	- - -	- - -
		1.0×10^6	5,5,8	11,11,32	14,14,22	- - -	- - -
		1.0×10^4H	7,7,7	15,- -	- - -	- - -	- - -
		1.0×10^4	5,5,5	15,15,19	32,32,-	- - -	- - -
		1.0×10^2H	- - -	- - -	- - -	- - -	- - -
		1.0×10^2	6,- -	18,31,-	- - -	- - -	- - -
5.5	0.9762	1.0×10^6H	8,12,12	33,47,47	- - -	- - -	- - -
		1.0×10^6	18,- -	- -	- - -	- - -	- - -
		1.0×10^4H	13,19,-	46,46,46	- - -	- - -	- - -
		1.0×10^4	15,19,25	46,61,-	- - -	- - -	- - -
		1.0×10^2H	- - -	- - -	- - -	- - -	- - -
		1.0×10^2	24,24,-	45,- -	- - -	- - -	- - -
6.0	0.9744	1.0×10^6	- - -	- - -	- - -	- - -	- - -
		1.0×10^4H	- - -	- - -	- - -	- - -	- - -
		1.0×10^4	- - -	- - -	- - -	- - -	- - -
		1.0×10^2H	- - -	- - -	- - -	- - -	- - -
		1.0×10^2	- - -	- - -	- - -	- - -	- - -

Table 12a. The effect of inoculum size and preheating upon outgrowth time in days of the K Type E Cl. botulinum strain incubated 90 days in TPSY medium with KCl to control a_w.

KCl conc. % (w/v)	a_w	Size of inoculum	Incubation temperature (C)				
			30	21.1	15.6	10	7.2
4.5	0.9797	1.0×10^6H	5,6,6	14,20,-	41,- -	- - -	- - -
		1.0×10^6	6,6,6	12,12,-	35,35,-	- - -	- - -
		1.0×10^4H	6,- -	- - -	- - -	- - -	- - -
		1.0×10^4	9,- -	13,- -	26,- -	- - -	- - -
		1.0×10^2H	- - -	- - -	- - -	- - -	- - -
		1.0×10^2	10, -	- - -	- - -	- - -	- - -
5.0	0.9776	1.0×10^6H	8,8,8	27,27,-	- - -	- - -	- - -
		1.0×10^6	8,8,8	25,- -	- - -	- - -	- - -
		1.0×10^4H	- - -	- - -	- - -	- - -	- - -
		1.0×10^4	13,13,19	40,40,-	40,- -	- - -	- - -
		1.0×10^2H	- - -	- - -	- - -	- - -	- - -
		1.0×10^2	- - -	- - -	- - -	- - -	- - -
5.5	0.9762	1.0×10^6H	- - -	- - -	- - -	- - -	- - -
		1.0×10^4H	- - -	- - -	- - -	- - -	- - -
		1.0×10^4	- - -	- - -	- - -	- - -	- - -
		1.0×10^2H	- - -	- - -	- - -	- - -	- - -
		1.0×10^2	- - -	- - -	- - -	- - -	- - -
6.0	0.9744	1.0×10^6	- - -	- - -	- - -	- - -	- - -
		1.0×10^4H	- - -	- - -	- - -	- - -	- - -
		1.0×10^4	- - -	- - -	- - -	- - -	- - -
		1.0×10^2H	- - -	- - -	- - -	- - -	- - -
		1.0×10^2	- - -	- - -	- - -	- - -	- - -

7.2 C, but in most cases spores with heat treatment required longer times to germinate and to produce outgrowth. With smaller inoculum sizes, irregular growth response and sometimes complete inhibition were observed, especially with the K strain. The heat shocked inoculum containing 1.0×10^2 spores/ml of this strain did not produce any growth in the 35% sucrose medium (a_w 0.9797), except for one tube which was positive after 28 days of incubation at 15.6 C (Table 10a). The spores without heat treatment produced outgrowth at 30 C in one tube in 13 days, at 21.1 C in two tubes in 26 days, and at 15.6 C in two tubes in 40 days of incubation. The inhibition and irregular response were more evident at the lower temperatures.

Outgrowth of the K strain was inhibited except for one tube when the 4.5% (a_w 0.9797) and 5.0% concentrations (a_w 0.9776) of KCl media were inoculated with 1.0×10^4 heat shocked spores/ml (Tables 12,12a). The heat shocked VH spores produced poorer outgrowth in this medium also. The 1.0×10^2/ml inoculum size was inhibited at all three concentrations tested, and prolonged lag times could also be observed.

This prolongation of the lag times or even inhibition was observed in the sodium formate medium, too, (Tables 11, 11a), especially with the K strain. Tubes receiving inocula containing 1.0×10^2 heat shocked spores/ml were negative even at 30 C, while the non heat shocked inocula produced positive tubes.

The temperature effect: Temperature definitely had an effect on the lag period as temperature became limiting for the bacteria. Lag time increased as the temperature decreased. As shown in Tables 10a and 11a, the 7.2 to 10 C incubation temperatures inhibited the growth of the K strain in the 4.5% KCl and Na formate concentrations (a_w 0.9797 and 0.9791), except for two positive tubes at 7.2 C in the sodium formate medium. The VH strain was also inhibited at these temperatures in the 4.5% KCl medium (a_w 0.9797, Table 12). The inhibitory effects of decreasing incubation temperatures produced erratic outgrowth when the size of the inoculum decreased. The K strain was more sensitive to the adverse effect of lower temperature than the VH strain, although in the KCl media both strains were inhibited at 10 and 7.2 C. In the sodium formate medium the VH strain produced outgrowth at these temperatures and the sucrose medium yielded more regular outgrowth.

These observations point out again that functional differences between the strains do exist.

Generally, a decrease in the size of the inoculum resulted in irregular outgrowth and inhibition at higher concentrations in all of the three media tested. When a decreased number of spores was inoculated into the media, inhibition was found at a_w levels previously found uninhibitory for the larger inoculum sizes.

Heat shock generally had an adverse rather than a beneficial effect on the spores of the strains tested. In

most cases the heat treatment delayed or even inhibited outgrowth at concentrations where spores without heat shock grew well. This adverse effect of heat was best demonstrated with the smaller (1.0×10^2 spores/ml and 1.0×10^4 spores/ml) inocula. There was some indication that heat may initiate outgrowth at concentrations otherwise inhibitory for growth and that the beneficial or adverse effect of the heat treatment depends on the strain involved.

The temperature effect became more defined at the lower incubation temperatures. At these temperatures the lag time was greatly increased and the rate of growth and the amount of cellular material was decreased.

SUMMARY AND CONCLUSIONS

The outgrowth of the K, A6247, VH and SF Type E Clostridium botulinum strains was studied in trypticase-peptone-sucrose-yeast extract (TPSY) medium with the addition of various concentrations of solutes. An inoculum size of 1.0×10^6/ml was inoculated into the TPSY medium and sucrose, sodium chloride (NaCl), potassium chloride (KCl), sodium formate and glycerol were added. The equilibrium relative humidity (ERH) of these media was determined with the graphical interpolation method and expressed as water activity (a_w). The molar concentrations and osmotic pressures of the various concentrations of solutes were calculated and related to the observed inhibitions. The inoculated samples were incubated at 30, 21.1, 15.6, 10 and 7.2 C. Outgrowth was observed in the concentrations of 35% sucrose (a_w 0.9766), 5.5% KCl (a_w 0.9762), 4.8% NaCl (a_w 0.9766) and 5.0% sodium formate (a_w 0.9761) and at lower concentrations but not in the 38.5% sucrose (a_w 0.9755), 6.0% KCl (a_w 0.9744), 5.0% NaCl (a_w 0.9754) and 5.5% sodium formate concentrations (a_w 0.9708).

The approximate osmotic pressure of the 38.5%, 1.12 molar (M) sucrose solution is 25.08 atmospheres. The 6.0% KCl and 5.5% sodium formate solutions are approximately 0.80 M and produce osmotic pressures of about 36.0 atmospheres,

while a 5.0% NaCl solution is 0.86 M and has an osmotic pressure of about 38.52 atmospheres.

No inhibition of growth was observed with the 15% glycerol concentration. The use of 15% glycerol lowered the a_w of the media to 0.9540, but did not inhibit outgrowth as observed with the other solutes. The 15% glycerol solution is approximately 1.63 M and has an osmotic pressure of about 36.5 atmospheres.

The existing difference in the inhibitory molar concentrations between the sucrose and salts solutions suggest that inhibition of outgrowth may not be explained by the molarity concept. However, the osmotic pressures of the inhibitory concentrations of sucrose and NaCl solutes were 25.08 and 38.5 atmospheres respectively, and the gas law equation is valid only at low solute concentrations. Osmotic pressure generally increases with higher sucrose concentrations much more rapidly than predicted by the simple equation used to calculate osmotic pressure. The inhibiting sucrose concentrations could have an osmotic pressure similar to those calculated for the salts. Therefore, the growth inhibiting characteristics of the solutes tested thus may be explained with the osmotic phenomenon if selective permeability is involved.

Since all the inhibitory salts and sucrose concentrations exhibited similar a_w values, the inhibition could also be explained by the a_w phenomenon and if inhibition was due to low a_w of the media tested then the inhibitory

level of growth for Type E strains in these solutions appears to be between 0.9708 and 0.9755 a_w.

Microscopic examination of the cultures showed that with sucrose in the medium the bacteria grew normally and only an increase of lag time was noticed. The rate of growth also was not affected and sporulation followed growth. In the salt-controlled media the cultures showed long filamentous vegetative cells, chains, some curved and a few straight rods. Sporulation was never observed. These differences in the electrolyte and non-electrolyte controlled media may indicate that the salts also affected the normal growth of the bacteria.

The effect of heat and the size of inoculum on outgrowth was also determined with the K and VH strains. Three spore inoculum sizes, 1.0×10^2/ml, 1.0×10^4/ml and 1.0×10^6/ml were inoculated into the a_w media with and without a heat treatment. Incubation temperatures were the same as in the previous experiment. Heat shocking was done for 15 minutes at 60 C. An increased lag time, irregular outgrowth and in some concentrations complete inhibition was observed with the smaller inoculum sizes at concentrations found not inhibitory for the larger inoculum sizes. In the 5% concentration sodium formate medium (a_w 0.9761) no outgrowth was observed with the 1.0×10^2/ml and 1.0×10^4/ml spore inoculum sizes, while the 1.0×10^6/ml produced good growth. In the KCl medium, the VH strain grew in the 5.5% concentration (a_w 0.9762) but the small 1.0×10^2/ml size inoculum produced irregular outgrowth. The outgrowth of

the K strain occurred only with the larger inoculum (1.0×10^6/ml) in the 5% concentration (a_w 0.9776). Inhibition was complete in the 5.5% concentration.

Both strains showed more regular outgrowth in the sucrose media with the higher inoculum, while with the smaller inoculum erratic response occurred.

When spores were heat shocked, an increase in lag time occurred especially with the smaller (1.0×10^2/ml) inoculum. A change in the inhibitory concentration was also observed; inhibition thus occurred at concentrations where spores without heat shock produced outgrowth.

Incubation temperature affected the lag time, the rate of growth and the amount of cellular material produced. As the incubation temperature was lowered the spores required longer times to germinate and a reduced number of total cells were produced.

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