

# Influence of Water Activity on the Growth of *Clostridium perfringens*

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Each of four strains of *Clostridium perfringens* was grown in modified fluid thioglycolate medium which was adjusted to yield selected water activity ( $a_w$ ) levels. The adjustments to secure the desired  $a_w$  levels were made with NaCl, KCl, or glucose. At each  $a_w$  level, further modification was effected to produce four pH values. Cultures were incubated at either 37 or 46 C. The solute used to achieve the reduced  $a_w$  levels appeared to have a definite effect on the magnitude of growth achieved, the rate of growth, and the limiting  $a_w$  at which growth would occur. Use of glucose as the controlling solute permitted growth at the lowest  $a_w$  level tested, 0.960, and yielded the greatest magnitude of growth as measured by turbidity values, at all of the  $a_w$  levels investigated. Cultures grown in the medium with added KCl generally demonstrated the longest lag times and the least amount of growth. Regardless of specific solute used, as the  $a_w$  level was lowered and the pH value decreased within each  $a_w$  level, the rate and amount of growth were lessened. It appeared, however, that low pH values had less effect on inhibiting growth at low  $a_w$  levels than at higher  $a_w$  levels. Those cultures incubated at 46 C generally exhibited shorter lag periods than those at 37 C, although the maximal growth attained was somewhat less than that achieved at 37 C. The response to all of the investigated conditions was similar for each of the four strains tested.

The concept of water activity ( $a_w$ ) as a measure of the moisture requirements of microorganisms has been emphasized by Scott (12, 13). In these review articles, he defined  $a_w$  as specifying the chemical potential of water in a solution relative to that of pure water at the same temperature and pressure and pointed out that it is numerically equal to the corresponding relative humidity expressed as a fraction. Wodzinski and Frazier (15) concurred in recommending the expression of the available moisture of the medium, in terms of  $a_w$ , as the most accurate means of describing the quantitation of substrate water accessible to the organism for growth or metabolic processes.

In a study in which two strains of *Clostridium perfringens* were employed, Kim (Thesis, Purdue University, Lafayette, Ind., 1965) reported the limiting  $a_w$  values for growth to vary from 0.93 to 0.97, depending upon the solute employed to control the  $a_w$  of the medium. She further reported that the  $a_w$  demands for the various cell functions of an organism were not necessarily the same. For example, she indicated that *C. perfringens* required a higher  $a_w$  level for spore production than for vegetative growth. These results were more recently reported in a paper

by Kang et al. (8). Baird-Parker and Freame (1), in a study dealing with *C. botulinum*, also noted a variation in  $a_w$  requirements predicated on cell function.

The present study had as its objectives: (i) determination of the limiting  $a_w$  for growth for four strains of *C. perfringens* type A in a complex medium, (ii) comparison of the effect on growth of each of three solutes used to lower the  $a_w$  of the basal medium, and (iii) investigation of the relationship of pH,  $a_w$ , and incubation temperature in the growth of *C. perfringens* vegetative cells.

## MATERIALS AND METHODS

**Cultures.** Four strains of *C. perfringens* type A were used throughout these investigations: ATCC 3624, originally received from the American Type Culture Collection; 215b, isolated from raw beef liver in this laboratory; NCTC 8238 (Hobbs type-2), acquired from the National Collection of Type Cultures; and IU1168, obtained from L. S. McClung. Neither strain ATCC 3624 nor 215b was known to have been associated with food-poisoning incidents, whereas strains NCTC 8238 and IU1169 were isolated from material associated with food-poisoning outbreaks.

**Media.** The following media were used during the

course of these investigations. (i) Cooked Meat Medium (CMM; Difco) was routinely employed to maintain the frozen stock cultures in the laboratory. (ii) Fluid Thioglycolate Medium (FTG; BBL) was utilized in growing vegetative cells from a stock spore inoculum. (iii) Sporulation medium (DS) of Duncan and Strong (5) was utilized for preparing spore suspensions basic to the experimental work. (iv) A modification of Fluid Thioglycolate Medium (FTG-1) served as the basal medium for the cells grown in media adjusted to selected  $a_w$  levels. FTG-1 was prepared from the same formula as FTG, omitting rezaurin, agar, and cystine.

**Preparation of inocula.** Spore suspensions were prepared in DS medium from the stock CMM cultures, as follows:

1 ml	→ 10 ml FTG	→ 0.1 ml	→ 10 ml FTG
CMM	16 hr		16–24 hr
		→ 1 ml	→ 100 ml DS
			24 hr

In those cases in which a strain apparently did not sporulate when the sequence just outlined was followed, an additional passage through DS medium, with an incubation period of 3 hr, was made before the final 24-hr incubation. All cultures were incubated at 37 C, and the resultant suspensions were checked microscopically for the presence of spores. The spores were stored in the spent DS medium at 2 C with no further treatment.

A 1-ml amount of the spore suspension was inoculated into 10 ml of FTG and heat-shocked for 20 min at 75 C. After incubating this FTG culture for 12 to 16 hr at 37 C, a transfer of 1 ml was made into 10 ml of fresh FTG, followed by incubation for 16 hr at 37 C. The latter FTG culture was centrifuged in a clinical centrifuge to obtain cells which were sufficiently packed so as to permit the supernatant liquid to be decanted. The cells were then resuspended in fresh FTG-1 that had been previously adjusted to yield the desired  $a_w$  level, and 0.15 ml of this suspension served as the inoculum for 150 ml of similarly adjusted FTG-1.

**Adjustment of  $a_w$ .** Three series of experiments were designed to follow the growth of each of the four strains of *C. perfringens* in media of progressively reduced  $a_w$  levels. For each series, the  $a_w$  adjustment was achieved with a different solute: NaCl, KCl, or glucose. Media were routinely prepared within 24 hr of use. No attempt was made to maintain a constant  $a_w$  level after inoculation of the medium, except that screw-capped glassware was routinely employed.

In series I, the medium was prepared by adding reagent grade NaCl to FTG-1 in predetermined amounts (grams of solute per 100 ml of FTG-1) prior to pH adjustment and autoclaving. FTG-1 with the added NaCl will be referred to in the remainder of this paper as FTG-N.

Series II utilized a similar procedure. Reagent grade KCl was added to FTG-1. This medium will be identified in this paper as FTG-K.

In series III, a two-step preparation was employed to adjust FTG-1 with glucose. The FTG-1 was made

double strength by use of a half volume of water. This double strength FTG-1 was adjusted for the specified pH values, allowing for a pH change during subsequent autoclaving and glucose addition. The predetermined amount of reagent grade glucose was dissolved in the remaining half volume of water. This glucose solution was sterilized by filtration using a Millipore filter (0.45  $\mu$ M pore size), and stored in sterile bottles. Prior to inoculation of the medium, equal volumes of the double strength FTG-1 and the glucose solution were aseptically combined. This medium will hereafter be designated as FTG-G.

**Determination of  $a_w$ .** A modified method of Daniels et al. (4) for freezing point determination was used to calculate the amount of solute necessary to achieve the desired  $a_w$  level in basal FTG-1. Two modifications were made: an alcohol bath was substituted for the air jacket, and crystallization was induced by addition of an ice crystal to the supercooled solution.

The "effective" gram moles of the solutes present in basal FTG-1 were determined from the freezing point of the basal medium established experimentally by use of the following equations:

$$n_2 = g/M_2 = G\theta/1,000 k_f \quad (1)$$

$$a_w = n_1/(n_1 + n_2) = P/P^\circ = N(\text{solvent}) \quad (2)$$

where  $M_2$  = the "effective" molecular weight of solute; this value represents the effect that the total solutes present have on the solvent in terms of the freezing point of the solution;  $g$  = the number of grams of solute added to 1,000 g of water in preparing the medium;  $G$  = grams of solvent used in medium preparation;  $K_f$  = molal freezing point depression constant, taken as 1.86 for water;  $\theta$  = freezing point depression measured in degrees centigrade;  $n_1$  = number of moles of solvent in the medium;  $n_2$  = number of moles of solute, from equation (1);  $P$  = vapor pressure of the solvent in solution;  $P^\circ$  = vapor pressure of pure solvent;  $N(\text{solvent})$  = mole fraction of solvent in solution.

The validity of using freezing point determination as an index of  $a_w$  lies in the relationship of the colligative properties of solutions, specifically between vapor pressure and freezing point. One form of Raoult's law states that the vapor pressure of the solvent in solution,  $P$ , is equal to the vapor pressure of pure solvent,  $P^\circ$ , multiplied by the mole fraction of the solvent in solution,  $N(\text{solvent})$ . Thus,  $P = P^\circ N(\text{solvent})$  or  $N(\text{solvent}) = P/P^\circ$ . By definition (equation 2),  $a_w = P/P^\circ$ ;  $N(\text{solvent})$  then is the only term needed to determine  $a_w$  by this procedure. Again, from equation 2,  $N(\text{solvent}) = n_1/(n_1 + n_2)$ . But from equation 1,  $n_2$  is found with the freezing point depression value, and  $n_1$  is known; i.e., the solvent is water whose molecular weight is 18, and, since  $G$  is known,  $n_1$  can be determined from the relationship  $n_1 = G/18$ . The fact that both equations are based on numbers of particles present and degree of particle interaction with the solvent establishes a valid relationship between freezing point depression and  $a_w$ .

**Adjustment of pH.** In determining the effect of interaction of variation in degree of acidity and  $a_w$  on the growth of *C. perfringens*, the media were adjusted as

TABLE 1. Solute added to medium (FTG-1) to attain the freezing point denoting selected  $a_w$  levels

$a_w$ Level	Solute					
	NaCl		KCl		Glucose	
	$\theta^a$	g/100 g of medium	$t^a$	g/100 g of medium	$\theta^a$	g/100 g of medium
0.995	0.47	— <sup>b</sup>	0.47	— <sup>b</sup>	0.47	— <sup>b</sup>
0.980	2.04	2.50	2.47	3.00	2.12	14.0
0.975	2.67	3.50	2.70	4.47	2.55	20.0
0.970	3.53	5.00	3.10	5.50	3.16	27.0
0.965	4.29	6.00	3.75	7.00	3.71	32.0
0.960						38.0

<sup>a</sup> Experimentally determined freezing point depression, C.

<sup>b</sup> Basal condition, no additional solute.

follows. We added 2 N NaOH or 2 N HCl in appropriate amounts to the respective unautoclaved FTG-1 media at predetermined  $a_w$  levels to produce the desired pH value ( $\pm 0.1$  pH unit deviation) after autoclaving. A Corning (model 10) pH meter was used to measure the pH of the media. Duplicate bottles of medium in 150-ml amounts were prepared. One bottle of medium provided samples used in checking for correct pH adjustment and for blanks in reading turbidity of the growing cultures; the second bottle served as the growth medium for the respective inocula. During the growth of the organism, no attempt was made to maintain a constant pH level by continuous buffering.

**Experimental design.** For all experiments, the growth of *C. perfringens* in the adjusted media was determined by turbidity readings for incubation periods to a maximum of 72 hr. A Klett Summerson photoelectric colorimeter (model 800-3) was used to determine turbidity, and the magnitude of growth was expressed in Klett units. Turbidity readings were made of the cultures at such intervals as to demonstrate best the end of lag, logarithmic growth, and beginning of death phases. Lag time in this paper refers to the time in hours for a culture to achieve a turbidity of 40 Klett units. Maximal growth is defined as the greatest turbidity reading recorded for a culture during the 72-hr growth period.

Each of the four strains of *C. perfringens* was grown in media adjusted to each of six  $a_w$  levels: 0.995 (basal), 0.98, 0.975, 0.970, 0.965, and 0.960 (Table 1). The  $a_w$  values 0.995 to 0.965 were calculated from equations 1 and 2 given above by using experimentally determined freezing point depressions; the solute concentration necessary to achieve an  $a_w$  level of 0.960 was extrapolated from a curve of  $a_w$  versus grams of solute added to achieve the freezing point depression corresponding to the  $a_w$  level. The 0.960  $a_w$  level was approximate because the plot of  $a_w$  versus solute concentration did not produce a linear curve because of

the high concentration of solute and resultant lack of an "ideal" solution.

During the course of the experiments, to permit the study of the possible relationships of  $a_w$ , pH, and temperature on the growth response of the organism, two incubation temperatures were tested (37 and 46 C). At the incubation temperature of 37 C, each of four pH values was established at each  $a_w$  level; these were 7.0, 6.5, 6.0, and 5.5. Each experiment consisted of a strain being grown in FTG-1 adjusted to a specific  $a_w$  by one solute and at each of four pH values. For the second incubation temperature, 46 C, only two strains of *C. perfringens*, one solute, and two pH values were tested. The two strains used were NCTC 8238 and IU1168, and they were each grown in FTG-G at pH 6.5 or 5.5.

For all experiments conducted during this investigation, a minimum of two replications was made.

## RESULTS

Except where noted, data presented are based upon average values expressed as turbidity readings for growth response for the four strains of *C. perfringens* employed throughout these experiments. Incubation of cultures was at 37 C except for one series of experiments which is delineated.

**Limiting  $a_w$ .** Growth curves for cultures of *C. perfringens* in FTG-1 adjusted to pH 6.5 at each of six  $a_w$  levels for each of three solutes are presented in Fig. 1(a-f).

The basal medium ( $a_w$  of 0.995, Fig. 1a) permitted the greatest degree of growth to develop with the shortest lag period. The limiting  $a_w$  for *C. perfringens* appeared to be 0.970 when FTG-N or FTG-K served as the medium for growth; growth continued to occur at approximately 0.960 when FTG-G was similarly employed. As demonstrated by turbidity values, decreasing the  $a_w$  level to 0.98 with each of three solutes resulted in a decrease in maximal growth attained in each case, as well as an increased lag period. Further lowering of the  $a_w$  level with each of the solutes progressively increased the lag periods and decreased the level of maximal growth attained. These findings were further substantiated by the growth curves resulting from the data compiled when the other pH values were tested. Thus, the response of this organism under these conditions reaffirms the observations made for many other species, that growth is influenced by the levels of  $a_w$  and tends to be proportional to the moisture available in the immediate environment.

**Environmental factors.** The particular solute used to reduce the  $a_w$  level of the medium appeared to affect both the magnitude of growth attained and the rate of growth. This influence of solute was evident at all  $a_w$  levels tested (Fig. 1,

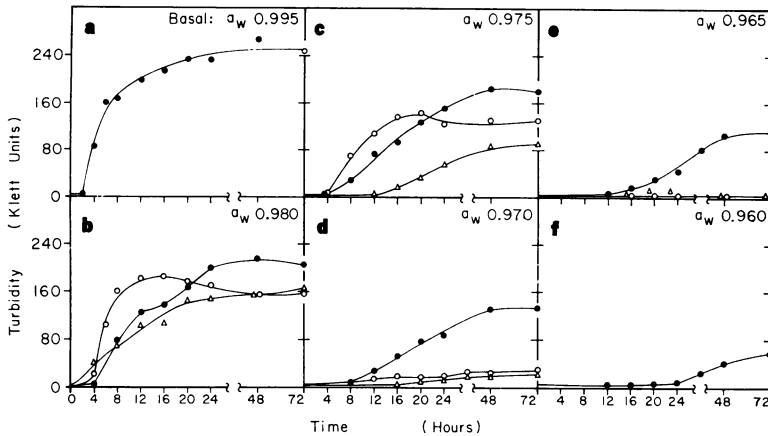


FIG. 1. Effect of varying  $a_w$  levels on the growth of *Clostridium perfringens* type A in FTG-1 medium, pH 6.5. Incubation temperature was 37 C. Adjustment of  $a_w$  was made with each of three solutes: NaCl (O), KCl ( $\Delta$ ), or glucose ( $\bullet$ ). Turbidity values express an average for four strains.

b-f). For example, at the  $a_w$  level of 0.98 maximal growth attained by the cultures grown in FTG-G was greater than that in FTG-N, but the cultures in FTG-N exhibited shorter lag times than those in FTG-G. The effect of solute used to reduce the  $a_w$  became more apparent as the  $a_w$  level was decreased and was especially evident in the range of limiting  $a_w$  for growth (Fig. 1e). No multiplication of the inocula was observed in the FTG-1 adjusted with either of the two electrolyte solutes at an  $a_w$  of 0.965, whereas growth of inocula continued to be evident in FTG-G at an  $a_w$  of 0.960. Once initiated, the rate and degree of growth achieved by cell inocula in FTG-G at at 0.960, the lowest  $a_w$  level tested, were greater than the rate and magnitude exhibited by cultures grown in FTG-N or FTG-K at  $a_w$  0.970.

Figure 2 (a-c) shows the effect of varying the degree of acidity on maximal growth attained by *C. perfringens*, when each of three solutes was used to adjust the  $a_w$  levels. As the pH value of the growth medium was lowered, growth response of *C. perfringens* continued, but at a level which seemingly was influenced by the degree of acidity in the environment. At any  $a_w$  level at which growth occurred in media adjusted to pH 7.0, growth also occurred in media of pH 5.5.

There was some indication that, as  $a_w$  was lowered, the consequence of the interaction between  $a_w$  and the variously adjusted pH values on maximal growth of the organism was reduced. The curves obtained when NaCl was used as a controlling solute (Fig. 3) suggest that, in media of high  $a_w$  levels, the effect of decreasing the pH level seemed more influential on limiting growth than in media of lower  $a_w$  levels; that is, at lower

$a_w$  levels the  $a_w$  level seemed to be more important in determining the amount of growth than was the low pH value. This decreased effect of pH value occurred with each of the three solutes. The fact that the acid or alkali added in adjusting the pH value would have some effect on the precise  $a_w$  value attained cannot be ignored, but the quantities involved were such as to suggest that any change would be negligible.

Although the growth response to different pH values within each  $a_w$  level, and to the  $a_w$  levels adjusted by each solute, varied with respect to maximal growth attained, the pattern was essentially the same. It may be stated generally that pH 6.5 permitted as great or a greater magnitude of growth as did pH 7.0. Cultures grown in media adjusted to pH 5.5 produced the lowest levels of growth. In those cases in which great differences in growth response to the four pH values were observed, it was between pH 5.5 and the other three levels.

When response to concurrent adjustments in  $a_w$  levels and pH values was examined in terms of lag time (Table 2), it was observed that, in FTG-N, as the pH value was decreased from 7.0 to 5.5 and as the  $a_w$  level was decreased at each pH value, there was an increase in lag time or no growth occurred. In FTG-N, at an  $a_w$  of 0.970, none of the pH values tested yielded a turbidity of 40 Klett units within the 72-hr observation period, and at an  $a_w$  of 0.965 no growth occurred at any of the pH values tested. The cultures grown in FTG-K and FTG-G reacted similarly to those in FTG-N, both demonstrating increased lag times to progressively decreased  $a_w$  and pH values.

Growth comparisons were made for two strains of *C. perfringens* (NCTC 8238 and IU1168) grown in FTG-G adjusted to pH 6.5 or 5.5 and incubated at either 37 or 46 C. Figure 4

shows a comparison of the maximal growth attained by the cultures incubated at 37 or 46 C in media adjusted to each of six  $a_w$  levels and two pH values. Maximal growth achieved by the cultures grown at 37 C exceeded the maximal growth at 46 C, except in the basal medium which was adjusted to pH 5.5. At all  $a_w$  levels tested, less growth was achieved in media adjusted to pH 5.5 and 6.5 when cultures were incubated at 37 C, whereas, for those cultures incubated at 46 C, pH 5.5 permitted slightly greater growth at two  $a_w$  levels (0.975 and 0.960) than pH 6.5.

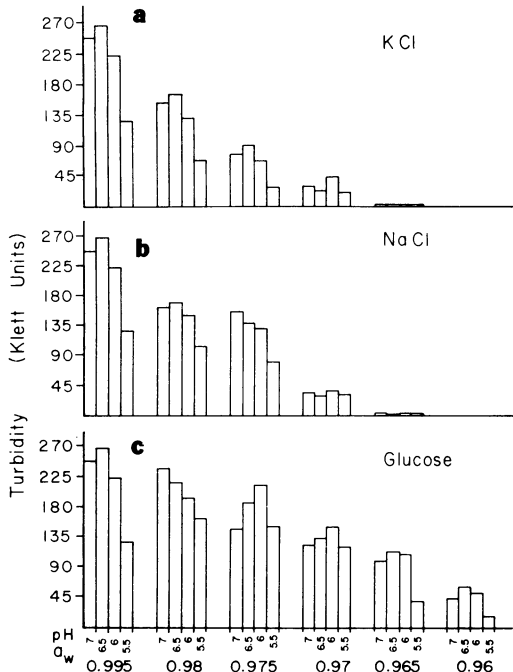


FIG. 2. Maximal growth of *Clostridium perfringens* type A grown in FTG medium adjusted to selected  $a_w$  and various pH levels (incubation at 37 C). Maximal growth is defined as the highest turbidity reading recorded during the 72-hr growth period, expressed in Klett units. Values presented represent an average for four strains. Adjustment of  $a_w$  was made with NaCl, KCl, or glucose.

Lag times are recorded for the two strains

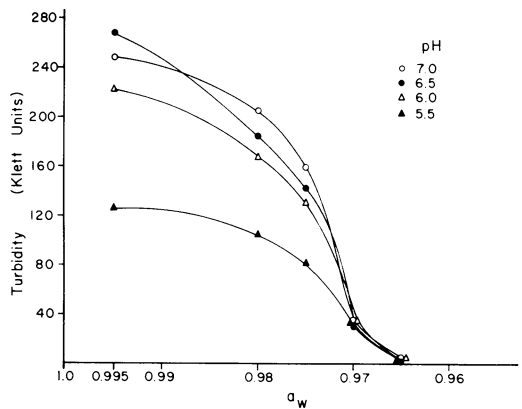


FIG. 3. Effect of pH on maximal growth attained by *Clostridium perfringens* type A grown in FTG-N adjusted to selected  $a_w$  levels with incubation at 37 C. Maximal growth is defined as the highest turbidity reading recorded during the 72-hr growth period, expressed in Klett units. Values presented represent an average for four strains.

TABLE 2. Lag time<sup>a</sup> for *Clostridium perfringens* type A grown in FTG-1 adjusted to selected  $a_w$  and pH levels (incubation at 37 C)

$a_w$ Level	pH 7.0			pH 6.5			pH 6.0			pH 5.5		
	FTG-N	FTG-K	FTG-G	FTG-N	FTG-K	FTG-G	FTG-N	FTG-K	FTG-G	FTG-N	FTG-K	FTG-G
0.995	3	3	3	3	3	3	5	5	5	8.5	8.5	8.5
0.980	4.5	6	5.5	4	3	6	6	10	9	10.5	17	16
0.975	5.5	18.5	7.5	6	21	8.5	8.5	30	12	15	- <sup>b</sup>	14.5
0.970	- <sup>b</sup>	- <sup>b</sup>	14	- <sup>b</sup>	- <sup>b</sup>	14	- <sup>b</sup>	57	17	- <sup>b</sup>	- <sup>b</sup>	30
0.965	NG <sup>c</sup>	NG	24	NG	NG	22.5	NG	NG	24	NG	NG	- <sup>b</sup>
0.960	- <sup>d</sup>	- <sup>d</sup>	69	- <sup>d</sup>	- <sup>d</sup>	48	- <sup>d</sup>	- <sup>d</sup>	47	- <sup>d</sup>	- <sup>d</sup>	- <sup>b</sup>

<sup>a</sup> Lag time is defined as the time in hours for cultures to achieve a turbidity of 40 Klett units. Values presented represent average for four strains.

<sup>b</sup> Turbidity achieved by the cultures did not attain a Klett reading of 40 units within the 72-hr growth period.

<sup>c</sup> No growth.

<sup>d</sup> Determinations not made.

incubated at 37 or 46 C in Table 3. In contrast to magnitude of growth, the cultures incubated at 46 C frequently demonstrated shorter lag times than those at 37 C, especially at the higher  $a_w$  levels. There was a marked trend for lag times to be lengthened as the  $a_w$  level was decreased for both incubation temperatures. Except for strain NCTC 8238 at an  $a_w$  of 0.975 and 46 C, both at 37 and 46 C, lag times were shorter for cultures grown in media adjusted to pH 6.5 than pH 5.5.

**Effect of strain.** Decreases in growth were

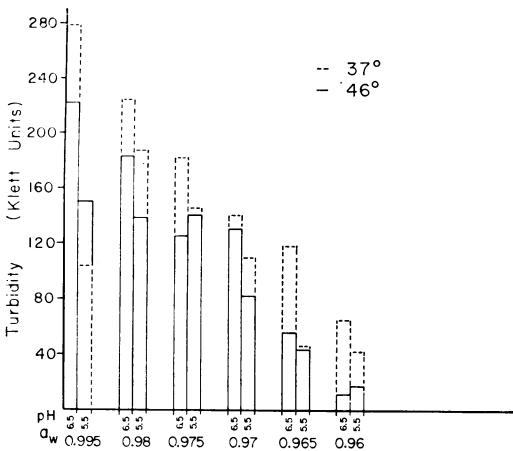


FIG. 4. Comparison of maximal growth of *Clostridium perfringens* type A grown in FTG-G with incubation at 37 or 46 C. Maximal growth is defined as the highest turbidity reading recorded during the 72-hr growth period, expressed in Klett units. Values presented represent an average for two strains, NCTC 8238 and IU1168.

observed fairly consistently as the  $a_w$  level was decreased at all pH values for all strains, although strain ATCC 3624 produced a slightly more variable pattern than the other strains. Furthermore, the effect of decreasing the pH value at each  $a_w$  level did not result in definitive difference in performance among the strains although there were some variations. Under the conditions of these experiments, with minor exceptions, the performance of the four strains was relatively uniform in terms of pattern of maximal growth. Also, when lag times of the respective strains were examined in terms of the effect of  $a_w$  and pH, the four strains responded similarly.

When the response of the individual strains to different temperature incubations is considered, IU1168 cultures grown at 46 C generally had shorter lag times than NCTC 8238 at the same temperature (Table 3). Similar comparisons at 37 C indicated that, in most cases, IU1168 again demonstrated shorter lag times than NCTC 8238. It is interesting to note that, when incubated at 46 C in media adjusted to pH 6.5, cultures of NCTC 8238 showed no growth at an  $a_w$  of 0.965 or 0.960, although growth occurred at these  $a_w$  levels in media adjusted to pH 5.5. At the lowest  $a_w$  levels tested, lag times were greater for IU1168 cultures incubated at 46 C than at 37 C; the NCTC 8238 cultures did not grow at 46 C in media adjusted to pH 6.5, but they did grow at 37 C.

**DISCUSSION**

The  $a_w$  levels of 0.995 to 0.96, which permitted various degrees of multiplication of cells for the four strains of *C. perfringens* tested here, fall within the range determined by Kim (Thesis,

TABLE 3. Lag time<sup>a</sup> for two strains of *Clostridium perfringens* type A grown in FTG-G<sup>b</sup>, adjusted to selected  $a_w$  and pH values, and incubated at 37 or 46 C

$a_w$	37 C				46 C			
	IU1168		NCTC 8238		IU1168		NCTC 8238	
	pH 6.5	pH 5.5	pH 6.5	pH 5.5	pH 6.5	pH 5.5	pH 6.5	pH 5.5
0.995	3.5	8.0	3.5	9.5	2	5	2.0	8
0.980	5.5	19.5	8	18	3	6	4.5	11
0.975	7	11.5	12	15.5	6	9	30	18.5
0.970	10	30	21	32	6	15	12	17
0.965	16.5	36	34	- <sup>c</sup>	25	72	NG <sup>d</sup>	37
0.960	38	58	37	- <sup>c</sup>	- <sup>c</sup>	- <sup>c</sup>	NG	- <sup>c</sup>

<sup>a</sup> Lag time defined as the time in hours for cultures to achieve a turbidity of 40 Klett units.

<sup>b</sup> Glucose used to adjust  $a_w$  values.

<sup>c</sup> Turbidity achieved by the cultures did not attain a Klett reading of 40 units within the 72-hr growth period.

<sup>d</sup> No growth.

Purdue University, 1965) and reported also by Kang et al. (8). In the instance in which the most direct comparison between the two studies is possible, i.e., control of  $a_w$  by use of NaCl, Kim observed growth at an  $a_w$  of 0.97 as did we; however, the subsequent  $a_w$  level tested by her was 0.95 and she found no growth; indeed, a rather rapid decrease in cell counts occurred. Our results indicate an  $a_w$  of 0.965 to be limiting for growth under similar conditions. The strains of *C. perfringens* utilized in the experiments were not the same in the two laboratories. A second difference was some variation in the amount of NaCl per volume of water employed in achieving the desired  $a_w$  control, and, third, the growth media to which the adjusting solute was added were not identical.

The question of the influence on growth of an organism of the solute used to adjust the  $a_w$  of the medium, other than an effect directly attributable to the alteration in  $a_w$ , is moot. Scott (12, p. 106) stated "...the biological response to a particular  $a_w$  was, at least, for some organisms largely independent of the type of solutes and the total water contents of the substrate." Support has been given to this view by other workers (6, 11, 15-17), although review of the evidence offered does in some cases suggest differences in minimal  $a_w$  levels, depending upon whether the solute added to the medium for control of  $a_w$  was salts or sugar. Kim (Thesis, Purdue, University, 1965) concluded that the growth response of *C. perfringens* to various degrees of  $a_w$  was not independent of the kind of solute. Her findings were supported by the results reported by Baird-Parker and Freame (1) for *C. botulinum*. 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 minimal  $a_w$  level required for the growth of the organism (Fig. 1).

It would seem quite reasonable that there should be a difference in response of the organism depending on whether nutritive or non-nutritive solute was used to adjust the  $a_w$  level of the medium. Scott (12) suggested that possibly only when certain minimum nutrient requirements were satisfied did limiting  $a_w$  for growth become independent of nutrient supply. Fuchs and Bonde (7), as a result of their study of the nutritional requirements, stated that *C. perfringens* was strictly dependent on carbohydrates and similar compounds for its energy. It can be hypothesized that, in the present study, glucose acted to increase the nutrient supply of the medium to the extent that greater growth was possible. In a preliminary unreported study in the present investigation, the pH value of the cultures was

recorded at various intervals during the 72-hr growth period. It was observed that at corresponding  $a_w$  levels those cultures grown in FTG-G reduced the pH value of their respective media to a lower pH value than did those cultures grown in FTG-N or FTG-K. The increase in acidity of FTG-G was probably due to the production of acids from the metabolism by the organism of the added glucose. No effort was made here to ascertain whether the growth of *C. perfringens* would have been altered if the pH of the medium had been buffered.

At lower  $a_w$  levels, the inhibitory effect of low pH values on growth appeared to be diminished (Fig. 3). This was especially noticeable when comparing the highest and lowest pH values tested (7.0 and 5.5). Wodzinski and Frazier (15-17) have reported that, in their investigations with *Pseudomonas fluorescens*, *Aerobacter aerogenes*, and *Lactobacillus viridescens* at non-optimal 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. An explanation for the difference in behavior of the organisms employed in this study and those of Wodzinski and Frazier might be that *C. perfringens* apparently does not have a single optimal pH value for growth. Rather, it was observed in the present study that cultures grown in FTG-1 adjusted to pH 7.0 or 6.5 gave similar growth rates and magnitudes with no consistent differences. Smith (14) studied the effect of pH on generation times with strains of *C. perfringens* type A and observed that there was no sharp optimal value between pH 6.0 and 7.5 for manifestation of shortest generation times. Fuchs and Bonde (7) reported an optimal pH range for growth of *C. perfringens* between pH 6.75 and 7.5 in a partially defined medium. The greatest differences in the response to the four pH values were observed here to be between pH 5.5 and the other three values. Perhaps then the lesser effect of pH value at reduced  $a_w$  levels observed in this study might be because the majority of pH values tested were not greatly divergent from the optimal range.

It should be emphasized that, in this investigation, growth was measured by turbidity readings, and the values reported for growth included both viable and nonviable cells. Lysis of cells is a possible explanation of the lower turbidity values recorded for the incubation at 46 C.

The four strains employed in this investigation, on the whole, responded similarly to reduced  $a_w$  and pH conditions. This homogeneity in response by strains of the same species is in agreement

with the findings of Scott (11) and Christian and Scott (3), who studied strains of *Staphylococcus aureus* and *Salmonella*, respectively. However, Ohye et al. (10) suggested, on the basis of their data with *C. botulinum* type E, that substantial differences in the minimum  $a_w$  levels of various strains might exist. The latter investigators based their conclusions on data which demonstrated a divergent growth rate response by each of four strains to lowered  $a_w$  levels as the temperature of incubation was reduced.

The effect of  $a_w$  level on the growth of *C. perfringens* as demonstrated in the present study confirms the observations made by many others of a relationship between the organism and its moisture requirements. Inherent in a consideration of limiting  $a_w$  levels is the whole spectrum of the microbial environment, especially the pH value and temperature of incubation. The significance of these factors lies in the fact that food, as a suspending medium, provides an environmental situation which combines various degrees of  $a_w$  and pH. In general, a food may be regarded as a more complex substrate than a liquid medium in terms of its gross physical structure. The textural characteristics of the food may provide protection to the organism from adverse environmental conditions. The composition of a food may in some cases have a decided bearing on the  $a_w$  level available to the microorganism. For example, as pointed out by Scott (13), foods with a high fat content have substantially reduced water contents.

The work of Christian and Scott (3) indicated that *Salmonella* could tolerate slightly lower  $a_w$  levels in a food menstruum than in liquid medium. Scott (11) reported that the lower limits of  $a_w$  for growth in dried meat, dried milk, and dried soup were similar to those in liquid media. Kim (Thesis, Purdue University, 1965) found that *C. perfringens* manifested longer lag periods and less growth when grown in a food substrate, even when the  $a_w$  levels of food and of the laboratory media were quite similar. Whether this apparent difference in response is attributable to the genera or to the varying technique employed in the experiments remains an open question.

The temperature at which the food is held, the pH, and  $a_w$  levels of the food all become important factors in determining the microbiological quality of a food. *C. perfringens* has been isolated from many food products of limited water content, including dried soup, sauce mixes, and spaghetti mixes (9). The presence of *C. perfringens* in these menstria of relatively low  $a_w$  levels indicates

the ability of the organism to remain viable under adverse environmental conditions and suggests that the method of handling the food after rehydration is indeed important to human health.

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