THE INFLUENCE OF BACTERIOPHAGE, ANTIBIOTICS, AND Eh ON THE LACTIC FERMENTATION OF CUCUMBERS

THE INFLUENCE OF BACTERIOPHAGE, ANTIBIOTICS, AND E_h ON THE LACTIC FERMENTATION OF CUCUMBERS

by

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INTRODUCTION

In many commercial fermentations contamination can be controlled by sterilization of the raw materials and by inoculation with pure cultures of the fermenting agents. The cucumber fermentation, on the other hand, depends upon the addition of relatively large amounts of salt to inhibit the development of undesirable micro-organisms. These undesirable micro-organisms, of which members of the genus Bacillus are probably the most important, are normal inhabitants of the soil and gain access to the fermentation vats through the small particles of soil adhering to the cucumbers at the time they are salted.

Each year the pickle industry suffers considerable losses due to spoilage. Usually this spoilage occurs in the latter stages of the fermentation, although in some instances spoilage is evident within the first few weeks after the cucumbers have been salted. During the past two years numerous samples of brine from spoiled salted cucumbers from all parts of the United States and Canada have been analyzed in this laboratory. The preponderance of aerobic spore-formers in many of these brines has suggested the need for further investigations.

Although a certain amount of attention has been devoted to the study of this particular group of organisms, there has been no attempt to define the conditions which determine their ability to overgrow the normal lactic acid organisms in certain vats and yet fail to develop in others under apparently similar conditions.

The purpose of this study is to investigate some of the factors which might explain the failure of the lactic fermentation of cucumbers

to proceed normally and thus permit these aerobic sporogenic bacteria to grow and in some cases cause spoilage.

LITERATURE REVIEW

Bacteriological investigations of spoilage in cucumber fermentations to date have dealt primarily with the isolation of a limited number of organisms from the brines of softened picles and with studies of their tolerance to salt and their abilities to cause softening of pickles.

As early as 1899 Aderhold (1) isolated spore-forming bacteria and Bacterium coli from softened pickles. This investigator, however, erroneously concluded that Bacterium coli was the cause of the softening and attached no significance to the presence of the spore-formers. Kossowicz (30), in 1908, similarly studied the softening of pickles and reported that the Bacillus mesentericus group was concerned with soft pickles and not, as reported by Aderhold, Bacterium coli.

Rahn (37), in 1913, conducted one of the earliest investigations of spoilage of pickles in this country. He recorded various factors contributing to spoilage of salt stock. Acid was found to be effective in inhibiting the growth of proteolytic bacteria while salt alone was of little value.

LeFevre (31) studied the ability of 50 organisms to attack vegetable matter and found that 16 of this number were capable of softening cucumbers. Only two organisms (B. vulgatus and B. mesentericus fuscus) were capable of growing in high salt concentrations and only four (B. vulgatus, B. carotovorus, B. cereus, and B. lactic aerogenes) could grow over a range of pH 7.0 to pH 4.0. LeFevre concluded that softening was caused by a variety of organisms and that 7-8 per cent salt was the critical point in the relationship of salt to the preservation of cucumbers.

Joslyn (27), in 1928, investigated the softening of dill pickles and agreed with previous workers that this type of spoilage was caused by bacterial action. He also observed that slipperiness increased in brines of pH 3.0 to pH 3.1 and suggested that the enzymes produced by spoilage organisms were probably active at a lower pH than that tolerated by the organisms themselves.

In 1938 Fabian and Johnson (13) isolated an organism from the brine of soft pickles which was capable of causing slipery pickles in 6-12 hours and mushy pickles in 12-24 hours. This organism which corresponded to B. mesentericus fuscus grew in salt concentrations up to and including 9 per cent salt.

Identification Studies

The primary purpose of this study was to attempt to explain the failure of some cucumber fermentations to develop normally. Therefore, only those samples whose brines, upon analysis, showed abnormally large numbers of aerobic spore-forming organisms were selected for study. Undoubtedly much spoilage of this type occurs, however only six such brines have been analyzed in this laboratory over the period of time covered by this work. Although none of the pickles from these samples showed visible signs of softening, spoilage was manifested by off-odors, unusually turbid brines, and improper curing. A summary of the pertinent data of these samples is presented in Table 1:

Table 1. Results of analyses of brines from spoiled pickles.

Sample number	pH	Per cent salt	Per cent lactic acid	Bacterial count	No. of isolates
B	4.70	5.4	0.53	*	4
C	3.30	3.8	0.75	3.5×10^3	3
E	3.39	12.1	1.10	17.0×10^4	5
F	3.42	16.1	0.53	15.0×10^2	2
G	3.47	19.8	0.53	11.3×10^3	9
H	3.30	3.1	1.00	14.0×10^3	6

* Not available.

When examined bacteriologically, these samples appeared to contain only Gram-positive spore-forming bacilli. Microscopic examination of colonial types on solid media suggested that several of these brines were practically pure cultures of a certain organism and several of these colonies were isolated for further study. In the remaining samples, two or three colonial types were recognized and several of each type were isolated. In all cases, each colony selected represented many similar colonies on the same plate and a total of 29 colonies were isolated and purified by repeated plating for further study. Microscopic examination proved all cultures to be aerobic sporogenic Gram-positive rods thereby placing them all in the genus Bacillus.

All 29 cultures were then subjected to a series of biochemical studies in order to identify them and thereby eliminate duplication of effort in future work. The biochemical tests used in this study were, for the most part, those suggested by Smith, Gordon, and Clark (38) as the most satisfactory tests for characterizing members of the genus Bacillus.

In addition, the tolerance of each organism to salt was determined. Since these organisms were cultivated for several months prior to this experiment on a medium devoid of salt, all strains were transferred daily in a liquid medium of the following composition:

Bacto-proteose-peptone	7.0 g
Bacto-beef extract	3.0 g
Sodium chloride, C. P.	50.0 g
Distilled water	1000.0 ml

After daily transfer in this 5 per cent sodium chloride broth for 10 days, the percentage of salt was increased by 1 per cent on each transfer until a concentration was reached at which each organism failed to grow.

The ability of these organisms to elaborate enzymes capable of softening cucumbers was determined by using the method of Fabian and Johnson (13). The organisms were grown for five days at 30°C in 500 ml wide-mouth Erlenmeyer flasks containing 350 ml of the beet-sugar molasses medium described by these workers. After this period of incubation, a sound pickle from salt stock was freshened to remove salt, washed in toluene, and placed in the flask in such a manner that the entire pickle was covered with the medium. The medium was then layered with toluene, incubated at 30°C, and examined at appropriate intervals for deterioration of the cucumber.

Results and Discussion

On the basis of biochemical studies, these organisms can be divided into six separate types or species as shown in Table 2. In work of this type it is difficult to definitely assign a specific name to a

Table 2. Characteristics of aerobic sporogenic bacteria isolated from spoiled cucumber pickle brines.

	Types					
	I	II	III	IV	V	VI
Fermentation of:						
Sucrose	+	+	+	+	+	+
Lactose	-	-	-	-	+	-
Maltose	+	+	+	-	+	+
Glucose	+	+	+	+	+	+
Fructose	+	+	+	+	+	+
Mannose	-	+	+	+	+	+
Galactose	-	+	-	-	-	+
Xylose	-	+	-	-	+	+
Arabinose	-	+	-	-	+	+
Raffinose	-	+	-	-	+	+
Rhamnose	-	-	-	-	-	-
Mannitol	-	+	+	+	+	+
Dextrin	+	+	-	-	+	+
Casein hydrolysis	+	+	+	+	+	+
Starch hydrolysis	+	+	-	-	+	-
Gelatin hydrolysis	+	+	+	+	+	+
Nitrate Reduction	+	+	+	-	-	-
Voges-Proskauer Reaction	+	+	-	+	-	+
Salt tolerance (per cent)	9	9	11	11	10	9
Softening of cucumbers	-	-	+	+	-	-
Reaction in litmus milk	*Red., **pept.	Red., pept.	Red., pept.	Red., pept.	Red., pept.	Red., pept.
Number of isolates	15	2	3	4	4	1

*Red. = reduced
**pept. = peptonized.

member of the genus Bacillus on the basis of the classification in the 6th edition of the Manual of Determinative Bacteriology (5). This classification is based on the work of Smith et al. (38) and is an attempt to consolidate the genus. For apparent reasons, this grouping of various species into a single new species is not always compatible with certain rather important characteristics of the organisms. For example, those organisms listed as type III in Table 2 correspond to the previous descriptions of Bacillus mesentericus fuscus even to their ability to soften pickles as reported by Fabian and Johnson (13). However, in the 6th edition of Breed's Manual, this organism is listed as a synonym of Bacillus subtilis although differing in several respects from the present description of Bacillus subtilis. The organisms listed as type II in the table, on the other hand, correspond very well to the description of Bacillus subtilis and do not soften pickles. One other important difference between these two groups of organisms is their tolerance to salt. Type III organisms will grow in salt concentrations up to and including 11 per cent while type II organisms do not tolerate above 9 per cent salt.

Those organisms grouped in type I appear to be the most prevalent in these brines. Slightly more than half (15) of the 29 organisms studied were classified as Bacillus cereus. This organism, although predominating, does not decompose pickles and does not grow in salt concentrations above 9 per cent.

Of the remaining three groups, only those organisms belonging to type V were classified positively. These organisms, which agree in all respects with Bacillus megatherium, are not responsible for the soften-

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ing of pickles. Types IV and VI correspond most nearly to descriptions of Bacillus vulgatus and Bacillus pumilus respectively. Three of the four organisms belonging to type IV proved to be capable of softening cucumbers while the single isolate of Bacillus pumilus did not.

Individual members of each of the six groups were selected for use in later studies.

The occurrence of bacteriophage races specific for the lactobacilli concerned with the cucumber fermentation.

The discovery of bacteriophage by d'Herelle (17) led to many attempts to utilize this lytic agent for therapeutic purposes. More recently, however, it has been discovered that bacteriophage plays a deleterious role in some industrial fermentation processes which depend upon the action of microorganisms. McCoy (33) discusses this unique environment for the propagation of bacteriophages which is offerred by the industrial use of microorganisms. Bacteriophage as the cause of "sluggishness" in the acetone-butanol fermentation is well-known in the patent literature but not in scientific literature. The origin of bacteriophage in an industrial plant is usually obscure and various control measures have been proposed.

More recently, attention has been directed toward the failure of starters in the cheesemaking industry. Whitehead and Cox (41) isolated bacteriophage from an aerated starter culture which had failed to develop acid. In a similar manner, Johns and Katznelson (26) in Canada demonstrated that the sudden stoppage of acid development in several experimental vats of Cheddar cheese was due to the activity of a polyvalent streptococcal bacteriophage. Although the starter was a mixture of organisms, stoppage was as abrupt as that in cases where single strain starters are used.

Whitehead and Hunter (42) established the presence of bacteriophage for lactic streptococci in the atmosphere of commercial cheese factories. Finely divided particles of whey emitted from a whey separator and whey-

contaminated dust were believed to be the principle vehicles for the air-borne phage. Hunter (22) stated that the amount of the initial infection determined the extent to which the presence of bacteriophage was made evident in the vat, a heavy infection being necessary to cause complete cessation of acid development.

In addition to air-borne contamination, several other sources of infection have been indicated. Whitehead and Hunter (43) demonstrated that whey-infected milk cans might be the cause of infected starters since the pasteurizing treatment of the cheese milk was not sufficient to destroy bacteriophage derived from infection of milk cans. Hunter (23) showed that some partially resistant strains of Streptococcus cremoris were able to carry phage in symbiotic association when subcultured daily in milk over long periods of time.

The effects of physical and chemical agents on the viability of bacteriophages for lactic streptococci have been the subjects of several investigations. Hunter and Whitehead (24) found that sufficient concentrations of hydrogen or hydroxyl ions would inactivate streptococcal bacteriophage but that their effects between pH values of 4 and 7 were negligible. Lactic acid in a concentration of 2.5 per cent was shown to destroy phage at room temperature in less than five minutes. A concentration of 1 per cent lactic acid was permanently effective in less than 24 hours. Sutton (39) demonstrated that ultraviolet light effectively destroyed bacteriophage under conditions likely to be encountered in cheese factories.

Hunter (21) studied the effect of temperature on the growth of several strains of Streptococcus cremoris and their appropriate bacterio-

phage. Phage races were shown to possess a wider diversity of reaction to temperature conditions than the homologous organisms. Some races failed to multiply at 37°C. Prouty (35) reported that several strains of bacteriophage under observation in the dry state were still active after being held for two years at room temperature.

The similarity between the "slow" starters in the cheesemaking industry, the "sluggish" fermentations in the acetone-butanol industry, and the "abnormal" fermentations in the pickle industry has suggested the possibility that bacteriophage might be responsible for the latter abnormality. For that reason, the following portion of this study consists of a survey of brine, soil, and water samples for the presence of bacteriophage specific for species of the genus Lactobacillus, particularly for those strains of ~~Lacto~~L. plantarum which are responsible for the cucumber fermentation.

Experimental

The organisms used in this survey consisted of 14 cultures of lactobacilli from various sources and 20 cultures which had been isolated from active cucumber fermentations. The 14 cultures from non-pickle sources (Table 3) were obtained from Dr. Carl S. Pederson, New York State Agricultural Experiment Station, Geneva, New York, and represented seven different species.

In view of the degree of strain specificity shown by Leuconostoc mesenteroides phage (32), 20 additional strains of lactic acid bacteria were isolated from actively fermenting brines and purified by repeated

Table 3. Lactobacilli from sources other than fermenting cucumbers.

Organism	Source
Lactobacillus buchneri	Tomato catsup
Lactobacillus brevis	
Lactobacillus plantarum	Vegetable salad
Lactobacillus plantarum var. rudensis	
Lactobacillus plantarum	Orla-Jensen
Lactobacillus buchneri	Molasses
Lactobacillus arabinosus	*A. T. C. C.
Lactobacillus casei	A. T. C. C.
Lactobacillus delbruckii	Kluyver
Lactobacillus brevis	Cameron
Lactobacillus sp.	Pineapple
Lactobacillus brevis var. rudensis	
Lactobacillus buchneri	Bread dough
Lactobacillus plantarum	Flour paste

*American Type Culture Collection

plating. All isolated were Gram-positive non-sporogenic rods which occurred singly, in pairs, or in very short chains. No attempt was made to identify these organisms, but it was assumed that all strains were probably Lactobacillus plantarum (Orla-Jensen) Bergey et al. as described by Pederson (34). This assumption was based on the work of Etchells and Jones (12) who identified 49 cultures of lactic acid bacteria isolated during the acid fermentation of salt stock cucumbers as this species.

The brine samples which were assayed for the presence of bacteriophage consisted of 10 samples of processed dill and salt stock brines (Table 4) from various sources and 12 samples of dill brine (Table 5) received from a Canadian company which lost practicelly their entire year's pack of genuine dills.

Table 4. Analyses of brines from spoiled processed dills and salt stock cucumbers.

Brine no.	Source of brine	pH	Per Cent salt	Per cent lactic acid
1	Processed dills	4.70	5.4	0.53
2	"	3.57	3.9	0.75
3	"	3.49	3.3	0.75
4	"	3.45	4.8	0.75
5	"	3.32	3.4	0.30
6	"	3.50	3.7	0.30
7	"	3.35	3.5	0.30
8	"	3.55	2.5	0.90
9	Salt stock	3.05	14.4	0.75
10	"	3.90	22.0	0.18

Table 5. Analyses of brine samples from 12 vats of spoiled genuine dills.

Brine no.	Source of brine	pH	Per cent salt	Per cent lactic acid
CC-1	Genuine dills	3.50	4.1	0.75
CC-2	"	3.48	4.7	0.60
CC-3	"	3.52	4.6	0.75
CC-4	"	3.55	4.5	0.75
CC-5	"	3.40	5.0	0.90
CC-6	"	3.50	4.7	0.68
CC-7	"	3.30	4.4	0.60
CC-8	"	3.60	4.6	0.90
CC-9	"	3.60	4.3	0.75
CC-10	"	3.60	4.5	0.75
CC-11	"	3.55	4.3	0.90
CC-12	"	3.90	4.8	0.68

In order to determine whether bacteriophage, if present at the beginning of the fermentation, might have been introduced into the vats by

water used in the salting process or by the cucumbers themselves, eight samples of Lake Ontario water were obtained from the Canadian company and four samples of soil were collected directly from cucumber hills on the Michigan State College Experimental Farm.

For the assay of brine and water samples the following technique was employed: Each sample was first filtered through a sterile Pasteur-Chamberland filter (L-5 porosity) and 1 ml of this sterile filtrate added aseptically to each of a series of tubes containing 9 ml of a medium of the following composition:

Glucose-Tryptone-Yeast Extract Broth

Bacto-tryptone	10 g
Bacto-yeast extract	5 g
Bacto-beef extract	3 g
Glucose	1 g
K_2HPO_4	1 g
Distilled water	1000 ml
pH	6.7

After addition of the filtrates, each tube was inoculated with one loop of a 24 hour culture of one of the lactic acid organisms. A control tube without the filtrate was inoculated with each organism. The tubes were observed after one and two days for lysis. If no growth occurred, the bacteriophage suspension was filtered through a sterile Pasteur-Chamberland filter, inoculated into another tube of the medium and seeded with the organism. If the suspension retained its lytic activity after four or five repetitions of this procedure, it was considered positive for bacteriophage against that particular strain of the organism.

A somewhat different technique was employed with soil samples.

A 500 ml Erlenmeyer flask containing 200 ml of glucose-tryptone-yeast extract broth was inoculated with one drop of a 24 hour culture of each organism to be tested. After an incubation period of 12 hours at 30°C, 10 grams of soil were added and the flask incubated for an additional 12 hours. After this period of preliminary enrichment, the supernatant was first filtered through paper and then through a sterile Seitz filter. The filtrates were then tested for the presence of bacteriophage according to the procedure described for brine and water samples.

Results and Discussion

Filtrates from the enrichment cultures of all soil samples suppressed growth of all of the 20 brine isolates of L. plantarum for a period of at least 48 hours. This ability to inhibit these organisms persisted through five filtrations, corresponding to a 1-100,000 dilution of the enrichment culture of a 1-2,000,000 dilution of the original soil sample. These dilutions were considered to be sufficient to eliminate any possibility that antibiotic substances originally present in the soil could have been carried over into the first filtrate in sufficient concentrations to inhibit growth of these organisms.

None of the 22 brine samples or six water samples gave evidence of the presence of bacteriophage. The fact that bacteriophage was not found in these brines is not surprising since only one of the samples had a pH value greater than 4.0. These results are in agreement with the findings of Hunter and Whitehead (24) who showed that streptococcal bacteriophage was inactivated by hydrogen ions when the pH value was less than 4.0.

The antagonistic action of aerobic sporogenic soil organisms toward the lactobacilli concerned with the fermentation of cucumbers.

The antagonism exhibited by certain species of microorganisms for other groups of microorganisms has been recognized by microbiologists for more than sixty years. Garré (15) pointed out as early as 1887 that the presence in the soil of bactericidal substances excreted by certain types of soil bacteria might be responsible for the rapid destruction of pathogenic organisms when they are added to the soil. On the suggestion of Garré that such substances might be valuable therapeutic agents, many unsuccessful attempts were made to isolate these substances.

After the discovery of sulfa drugs, interest in these substances produced by soil organisms was revived. Logically enough, emphasis has again been placed on their value as therapeutic agents. Dubos (8) (9) (10) in a series of papers described the isolation of an alcohol soluble, water-insoluble substance from broth cultures of an aerobic sporulating soil bacillus corresponding to Bacillus brevis. This substance which was called tyrothrycin was separated into two fractions: (a) tyrocidine, which was bactericidal in vitro for Gram-positive and Gram-negative organisms; and (b) gramicidin, which was effective against only Gram-positive organisms.

Ark and Hunt (2) isolated two soil organisms, one of which was identified as Bacillus vulgatus, while the other, a yellow spore-bearing bacillus, was not identified. These organisms were found to be antagonistic in both liquid and solid media to a number of Gram-positive and

Gram-negative organisms including Streptococcus lactis, Leuconostoc mesenteroides, and Lactobacillus acidophilus.

Katznelson (28) isolated an aerobic spore-bearing bacillus which produced an antibiotic substance in a potato-dextrose-peptone medium. This substance was found to inhibit 77 out of 81 species tested. The majority of streptococci, staphylococci, lactobacilli, and clostridia were inhibited by this toxic principle while all Gram-negative organisms were unaffected.

Jansen and Hirschmann (25) reported that subtilin, an antibiotic substance produced by Bacillus subtilis on a medium consisting of mineral salts, sucrose, asparagine, and trace elements, was active against Gram-positive organisms. Foster and Woodruff (14) described another antibiotic principle produced by a soil isolate of Bacillus subtilis. This substance, which they called bacillin, was highly active against both Gram-positive and Gram-negative bacteria.

Excellent reviews on this subject have been written by Waksman (40), Hotchkiss (20), and Hoogerheide (19). These reviews thoroughly cover such aspects as the isolation, purification, chemistry, and physiological properties of tyrothrycin, tyrocidine, and gramicidin.

Experimental

A simple "cup" method was used for determining the ability of the aerobic sporogenic organisms used in this study to elaborate antibiotic substances against lactic acid organisms. Approximately 15 ml of glucose-tryptone-yeast extract agar was added to a sterile petri dish which had

been seeded with 1 ml of a 1-10,000 dilution of a 24 hour broth culture of the lactic acid organism. After the plates had been mixed and allowed to harden, sterile glass rings (8mm high X 16 mm diameter) were heated slightly in the flame and placed on the surface of the agar. In order to complete the seal between the ring and the agar, a thin layer of sterile 3 per cent agar was poured over the surface. To the cup thus formed was added 0.5 ml of a 72 hour culture of the spore-former which had previously been filtered through a sterile Pasteur-Chamberland filter.

The plates were incubated upright at 30°C and examined at 24 and 48 hours. The degree of inhibition was recorded as the maximum distance, expressed in millimeters, which inhibition extended beyond the inner edge of the ring.

The lactic acid organisms used in this study were comprised of one strain of Leuconostoc mesenteroides, six species of lactobacilli, all of which had been isolated from sources other than pickle brines, and ten strains of Lactobacillus plantarum which had been isolated from actively fermenting cucumbers.

The influence of the composition of the medium on the elaboration of antibiotic substances was determined by using four different media for growing the sporeformers. These media were: (a) Proteose-peptone broth consisting of 7 grams of proteose-peptone and 3 grams of beef extract per liter; (b) Glucose-tryptone-yeast extract broth; (c) Beet sugar molasses broth which was prepared by diluting a mixture of 100 grams of beet sugar molasses, 10 grams urea, and 10 grams of monobasic ammonium phosphate to

5° Brix and adjusting to pH 7; and (d) Cucumber extract which was prepared by mincing 200 grams of peeled cucumber and 1000 ml of distilled water in a Waring Blendor, digesting in an Arnold sterilizer for one hour, and filtering.

Results and Discussion

Of the seven lactic acid organisms which were isolated from sources other than brines, only three showed any degree of inhibition by any of the filtrates (Table 6). Of these three organisms, Leuconostoc mesenteroides, Lactobacillus plantarum, and Lactobacillus arabinosus, the latter two are now regarded as synonomous and certain strains of L. plantarum are believed to be the principle organisms concerned with the fermentation of cucumbers. L. mesenteroides, on the other hand, is not regarded as an important factor in the cucumber fermentation, but is responsible for inaugurating the sauerkraut fermentation.

Entirely different results were obtained when the filtrates were tested against the ten lactic acid organisms isolated from brines (Table 7). The most consistent results were obtained when the spore-formers were grown in cucumber extract. In this medium only B. mesentericus fuscus (C-1, H-1) and B. vulgatus (G-8, H-6) elaborated antibiotic principles which were active against the lactobacilli. Two isolates of each of the organisms were strongly inhibitory to all of the lactic acid organisms. These results are perhaps more significant in view of the fact that these species were the only ones which were capable of softening cucumbers.

When grown in glucose-tryptone-yeast extract broth, only two of

Table 6. The antagonistic action of aerobic sporogenic organisms toward strains of lactic acid bacteria isolated from sources other than pickle brines.

	Type I		Type II		Type III		Type IV		Type V		Type VI
	B-1	B-2	F-1	F-2	C-1	H-1	G-8	H-6	E-1	E-5	G-7
L. casei	-	-	-	-	-	-	-	-	-	-	-
L. arabinosus	-	-	±	±	-	-	±	±	-	-	-
L. brevis	-	-	-	-	-	-	-	-	-	-	-
L. plantarum	-	-	-	-	-	-	±	±	-	-	-
L. delbrueckii	-	-	-	-	-	-	-	-	-	-	-
L. buchneri	-	-	-	-	-	-	-	-	-	-	-
L. mesenteroides	±	±	±	±	±	±	±	±	±	±	±

- = No inhibition

± = Incomplete inhibition, does not extend beyond ring.

Table 7. Showing the effect of the composition of the medium on the elaboration of antibiotic substances against the lactic acid organisms isolated from cucumber brines.

(a) Cucumber extract

	Type I		Type II		Type III		Type IV		Type V		Type VI
	B-1	B-2	F-1	F-2	C-1	H-1	G-8	H-6	E-1	E-5	G-7
5-10	-	-	-	-	*15	20	*6	15	-	-	-
5-16	-	-	-	-	15	20	6	20	-	-	-
5-1	-	-	-	-	15	20	4	20	-	-	-
5-30	-	-	-	-	12	20	4	20	-	-	-
5-25	-	-	-	-	15	20	8	20	-	-	-
6-18	-	-	-	-	15	20	6	15	-	-	-
6-26	-	-	-	-	15	20	8	15	-	-	-
6-25	-	-	-	-	12	20	4	20	-	-	-
6-22	-	-	-	-	15	20	6	15	-	-	-
6-1	-	-	-	-	15	20	6	20	-	-	-

(b) Glucose-tryptone-yeast extract

	Type I		Type II		Type III		Type IV		Type V		Type VI
	B-1	B-2	F-1	F-2	C-1	H-1	G-8	H-6	E-1	E-5	G-7
5-10	-	-	-	-	-	-	-	-	-	-	-
5-16	-	-	-	-	-	-	-	-	-	-	-
5-1	-	-	-	-	-	-	-	-	-	-	-
5-30	-	-	-	-	-	-	-	-	-	-	-
5-25	±	±	±	±	-	-	-	-	-	-	-
6-18	-	-	-	-	-	-	-	-	-	-	-
6-26	-	-	-	-	-	-	-	-	-	-	-
6-25	-	-	-	-	-	-	-	-	-	-	-
6-22	-	-	-	-	-	-	-	-	-	-	-
6-1	-	-	-	-	-	-	-	-	-	-	-

See end of table for legend.

Table 7. (Continued)

(c) Proteose-peptone broth

	Type I		Type II		Type III		Type IV		Type V		Type VI
	B-1	B-2	F-1	F-2	C-1	H-1	G-8	H-6	E-1	E-5	G-7
5-10	-	-	+	+	-	-	±	-	-	-	±
5-16	+	+	+	+	+	+	+	+	+	+	+
5-1	+	+	+	+	-	-	-	-	+	+	-
5-30	7*	9	+	+	±	-	+	+	7	9	+
5-25	-	-	+	+	+	+	+	+	-	-	±
6-18	-	-	6	7	7	6	-	-	-	-	-
6-26	-	-	5	7	6	6	-	-	-	-	+
6-25	-	-	7	6	7	6	±	+	-	-	+
6-22	-	-	+	+	+	+	±	±	-	-	-
6-1	-	-	+	+	+	+	-	-	-	-	-

(d) Beet sugar molasses broth

	Type I		Type II		Type III		Type IV		Type V		Type VI
	B-1	B-2	F-1	F-2	C-1	H-1	G-8	H-6	E-1	E-5	G-7
5-10	+	+	+	+	+	+	+	+	4	-	+
5-16	-	-	-	-	+	+	9	6	4	4	9
5-1	-	-	-	-	+	+	-	-	-	-	-
5-30	-	-	-	-	-	+	7	6	6	9	6
5-25	-	-	-	-	5	6	3	4	-	-	-
6-18	-	-	-	-	+	+	-	-	-	-	-
6-26	-	-	-	-	+	-	-	-	5	5	+
6-25	-	-	-	-	-	-	2	+	5	5	+
6-22	-	-	-	-	+	+	-	-	-	-	-
6-1	-	-	-	-	5	6	±	-	2	2	±

+ = Inhibition only under ring; ± = Inhibition incomplete and only under ring; - = No inhibition.

* = Denotes maximum distance in mm that inhibition extends beyond ring.

the organisms, B. subtilis (F-1, F-2) and B. cereus (B-1, B-2), gave any indication of antibiotic activity and this was very slight and against only one strain of the lactobacilli.

The results obtained with proteose-peptone broth and the beet sugar molasses medium were less clear-cut. All of the spore-formers showed an antagonistic action toward some of the lactobacilli in both of these media, although in the beet sugar molasses medium, B. subtilis and B. cereus were active against only one of these organisms.

The relationship between the metabolic activity of aerobic sporogenic bacteria and their ability to grow in cucumber brines.

The first observation that certain groups of bacteria were characterized by definite reduction potentials exhibited in their cultures was made by Gillespie (16). He noticed the markedly different final potentials which developed in cultures of strict aerobes, as contrasted with those of facultative anaerobes, and suggested that these differences may apply generally to the two distinct groups of bacteria, aerobes and anaerobes.

Quastel and Stephenson (36) found that the presence of cysteine, or other reducing substances, in tryptic broth would induce good aerobic growth of Clostridium sporogenes. They suggested that the latent period exhibited by oxygenated Cl. sporogenes cultures was the time of incubation required for the non-proliferating cells to produce a certain minimum quantity of reducing substances in the medium. This minimum quantity of reducing substances, it was believed, was necessary in order to establish a limiting reduction potential for proliferation of the organism. In a medium which maintained a higher potential proliferation of the anaerobe could not occur. Allyn and Baldwin (3), observing the effect of cysteine on the zones of growth of Rhizobia in agar shake cultures, arrived at similar conclusions. In addition, they believed that the bacteriostatic effect of certain oxidizing and reducing substances was due in part to a "poising" of the medium at a potential unfavorable for growth. Sustaining this theory was the previous work of Cannan et al. (6) which showed that halogens lost the greater part of their toxicity when added under

conditions which prevented the attainment of oxidation-reduction potentials more positive than those of the indophenols.

Allyn and Baldwin (4) measured the time-potential curves of Rhizobia in two basic media: (a) mannitol-yeast water and (b) mannitol-nitrate broth. The former medium was more reducing in nature and supported growth at higher dilutions of the inoculum. Mannitol-nitrate broth permitted growth in similar dilutions only after the potential had been reduced.

Knaysi and Dutky (29) demonstrated that very low potentials also had an inhibitory effect on the growth of another aerobic organism, Bacillus megatherium. These workers lowered the potential of a meat infusion broth to -0.25 volts with 0.27 per cent sodium sulfite and inhibited the growth of this organism.

The relationship between the bacteriostatic action of certain indicator dyes and their capacities to poise media at definite oxidation-reduction potentials has been investigated by Dubos (7) and Wood, Wood, and Baldwin (44). Dubos, working with pneumococci and human and bovine strains of hemolytic streptococci, found that methylene blue and oxidized indophenols were bacteriostatic while the indigoes, malachite green, and litmus were not toxic. Since methylene blue and the indophenols were no longer bacteriostatic when present in the reduced form, Dubos concluded that the "inhibiting" dyes poised the medium at an oxidation-reduction potential outside of the range in which the inhibited organisms could grow. Wood et al. obtained similar results with Bacillus megetherium. The oxidized forms of indicators more positive than methylene blue inhibited this organism for 24 hours while those dyes possessing more negative potentials did not.

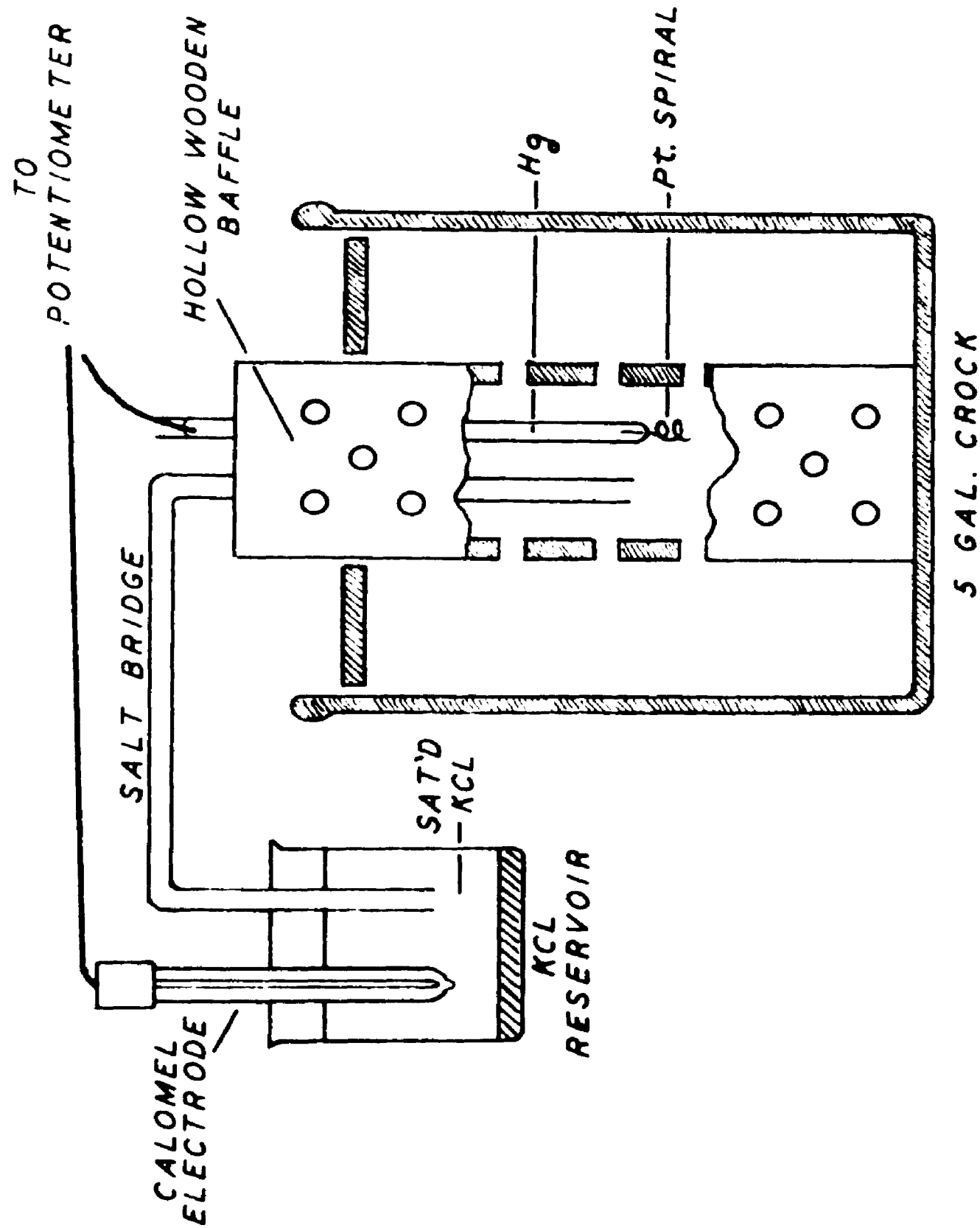
The influence of potential on the bacteriostatic action of dyes has suggested a possible explanation for the proliferation of bacterial cells in high brine concentrations. Hof (18), for example, was able to increase the salt tolerance of soil organisms from 6 to 18 per cent by adding sterile garden soil to the culture medium. Similarly, Fabian and Johnson (13) found that Bacillus mesentericus fuscus grew in a higher concentration of salt when grown in a medium containing beet sugar molasses. Doubtlessly the alteration of some physical property of the medium such as the oxidation-reduction potential enabled these workers to decrease the sensitivity of these organisms to salt.

In order to study the influence of salt upon sporogenic aerobic bacteria and the lactobacilli commonly found during cucumber fermentations, a series of experiments was conducted to study the oxidation-reduction potentials of these organisms in the presence of different quantities of salt at various time intervals.

1. Determination of normal time-potential curves of 16° and 40° salometer brines.

Washed cucumbers were placed in eight 5 gallon crocks. Half of the crocks were salted at 40° salometer and the remainder at 16° salometer. Platinum electrodes, prepared by sealing a bright platinum spiral into glass tubing, were immersed to a depth of approximately 8 inches below the surface of the brine. These electrodes together with KCl agar bridges were protected from the cucumbers by means of hollow wooden baffles (Fig. 1) through which numerous holes had been drilled to permit free circulation of the brine.

Fig. 1. Apparatus used for determining time-potential curves of fermenting cucumbers.



Oxidation-reduction potentials were measured at 0, 2, 4, 6, 9, 12, and 16 days by means of a Model G Beckman potentiometer. At the same time, samples were withdrawn for pH determinations and bacterial counts. Since all of the aerobic sporogenic organisms which had been previously isolated from brines were peptonizers, nutrient agar containing 5 per cent skim milk was used as the plating medium and only those colonies which hydrolyzed the casein were counted.

Results and Discussion

An analysis of the time-potential curves of fermenting cucumbers (Fig. 2a,b,c, and d and Fig. 3a,b,c, and d) indicates that there are no significant differences resulting from the two different salting procedures. The outstanding characteristic, and one which is common to both salt treatments, is the intensity of the reducing conditions which are established within the first few days after the cucumbers are salted. The fact that this sharp drop in potential coincides with the rapid evolution of gas suggests that the same organisms may be responsible for both of these phenomena. On the other hand, the simultaneous increase in the number of peptonizers offers another explanation for the potential drop since most of the members of the genus Bacillus which have been studied in pure culture have been shown to be of a reducing nature. If the first supposition is true, then it is possible that the peptonizers are able to actively proliferate in these high salt concentrations by virtue of the reduced potential.

Fig. 2a,b,c and d. Normal oxidation-reduction potential, pH, and peptonizer curves of cucumbers salted at 40° salometer.

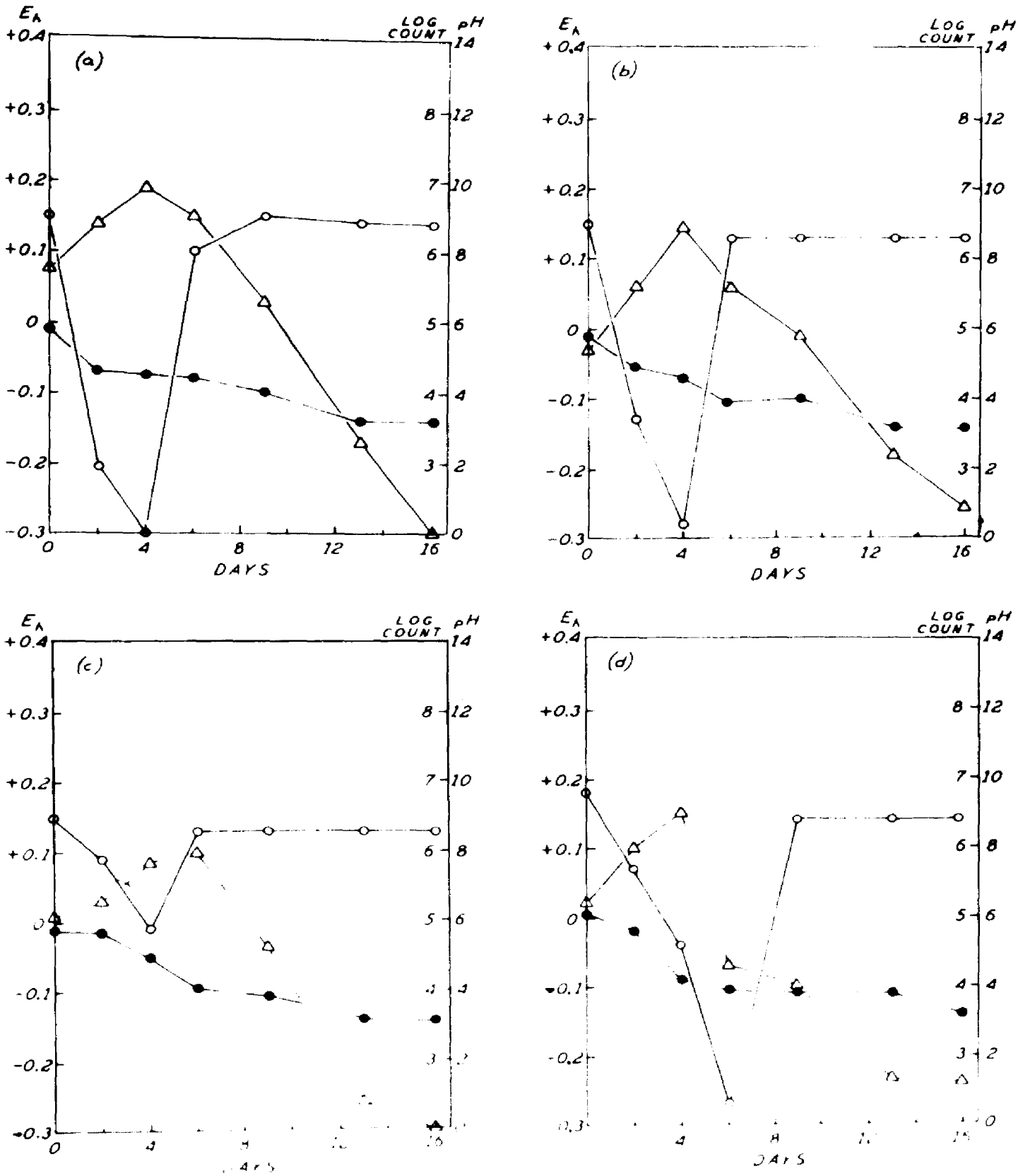
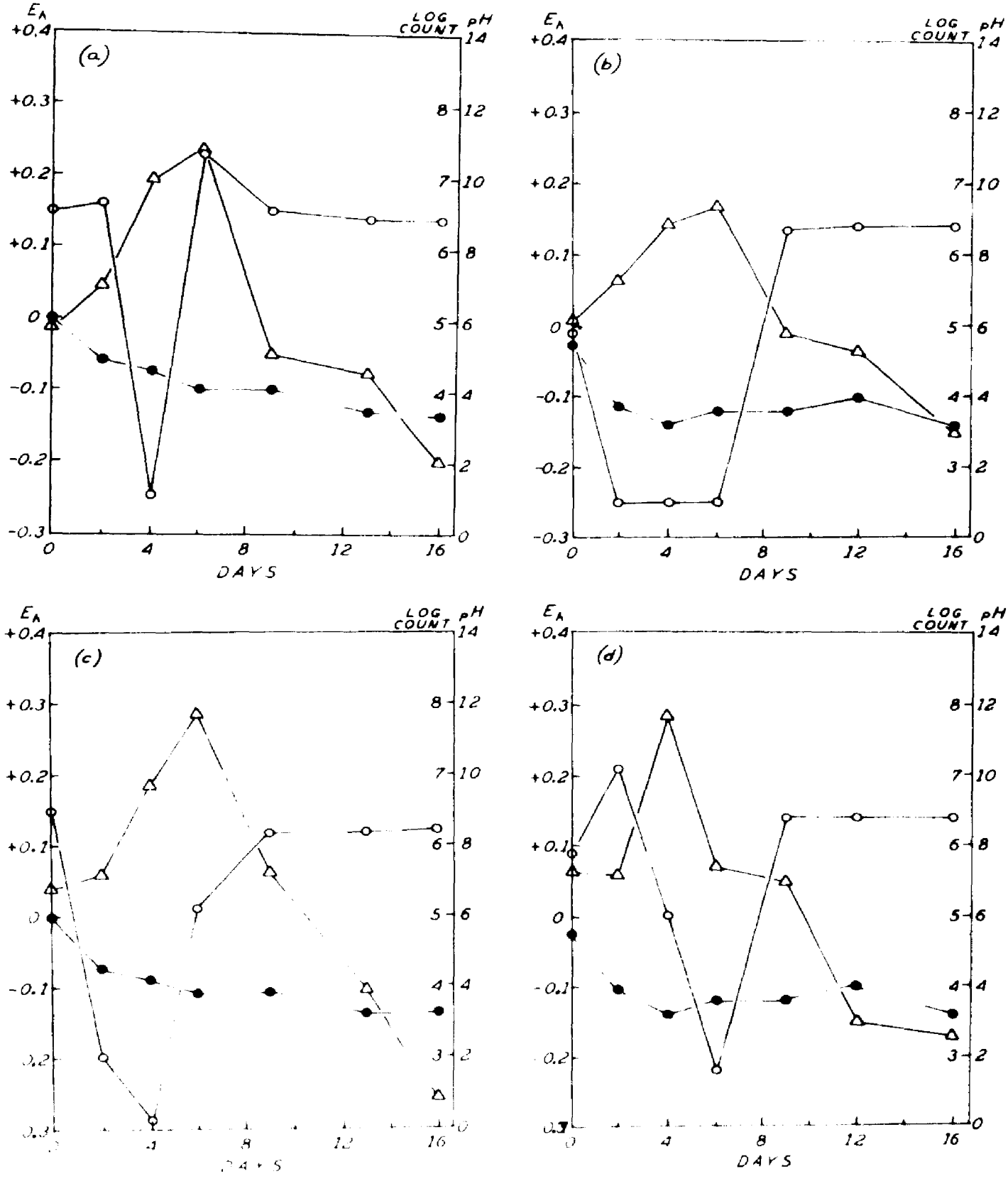


Fig. 3a,b,c and d. Normal oxidation-reduction potential, pH, and peptonizer curves of cucumbers salted at 16° salometer.



○ ○, E_h; ● ●, pH; △ △, LOGARITHM OF PEPTONIZERS PER MILLILITER

At any rate, under the conditions of this experiment, the predominance of the peptonizers was only transitory and they decreased in numbers as the acidity of the brines increased.

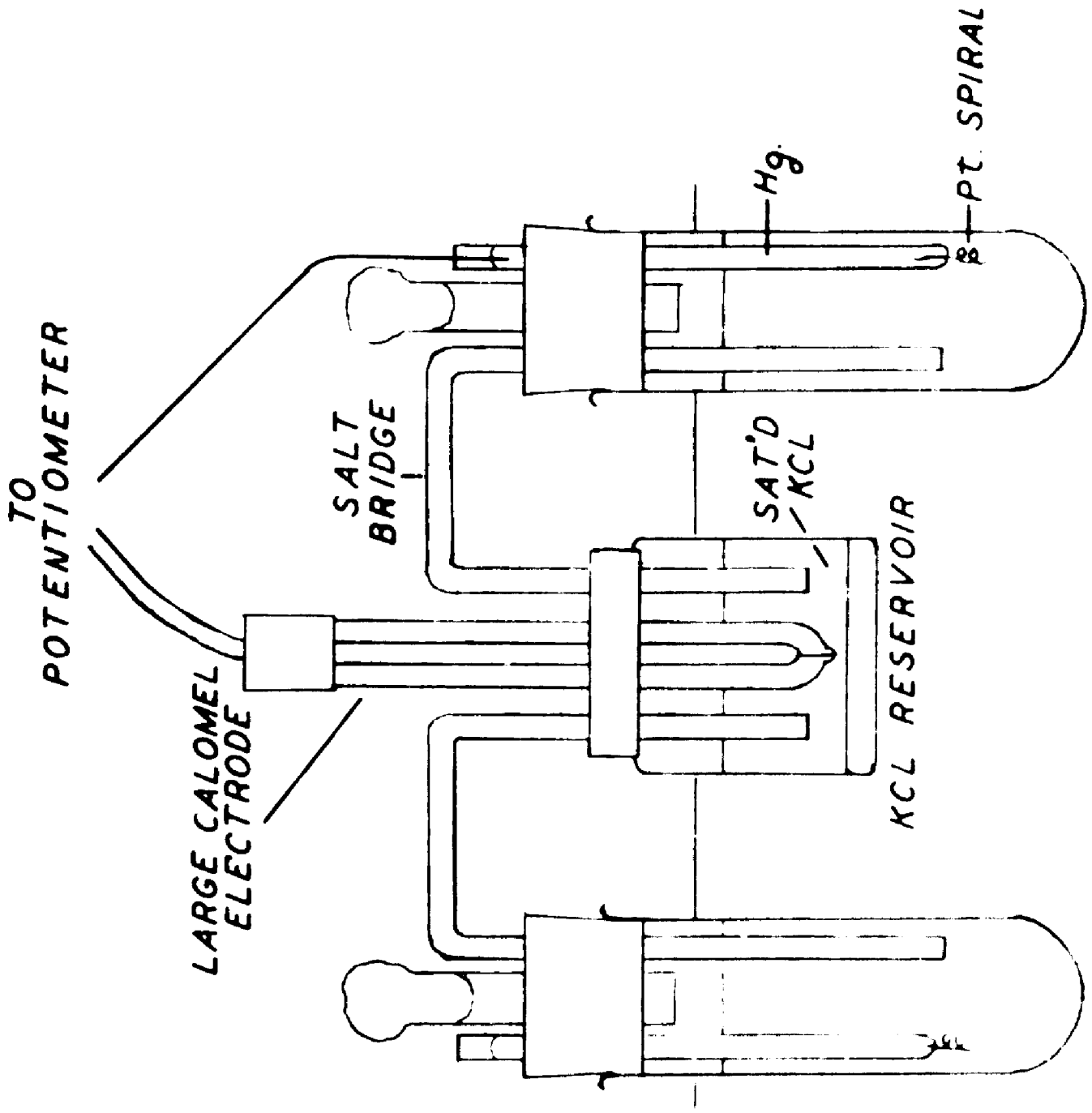
2. The determination of time-potential curves of some of the organisms from cucumber brines.

The apparatus which was used for determining time-potential curves of pure cultures in this and following experiments is shown diagrematically in Fig. 4. The individual cells consisted of pyrex test tubes (1 in. X 4 in.) equipped with three-hole rubber stoppers containing a platinum electrode, salt bridge, and a cotton-plugged tube through which the cells could be inoculated. A battery of six of these cells were arranged so that electrical contact between the cells and a common KCl reservoir could be established by means of salt bridges. Contact between the KC l reservoir and the potentiometer was made with a large calomel electrode. Each cell contained 20 ml of medium and the entire assembly was immersed to the level of the medium in a water bath controlled at 30°C.

After sterlization, the cells were incubated for at least 24 hours before inoculation to permit stabilization of the potential. The inoculum was 1 ml of a 1-100 dilution of a 24 hour broth culture of the test organism.

Normal time-potential curves were determined for the six species of sporogenic organisms. In addition to these, one strain each of Lactobacillus plantarum and Aerobacter aerogenes were used. Several attempts were made to isolate the strain of Aerobacter reported by Etchells

Fig. 4. Apparatus for determining time-potential curves of pure cultures.



Fabian, and Jones (11) as the causative agent of the early gaseous fermentation of cucumbers. All attempts, however, were unsuccessful.

The two media used in this study were proteose-peptone broth and cucumber extract.

Results and Discussion

One of the most significant features of the time-potential curves of these organisms is the non-reducing nature of Lactobacillus plantarum (Fig. 6c) as compared to Aerobacter aerogenes (Fig. 6d) and the six species of sporogenic organisms (Fig. 5a,b,c and d and Fig. 6a and b). Aerobacter, on the other hand, establishes intense reducing conditions in both media. This observation is particularly interesting since this organism is very closely related to Aerobacter cloacae which is responsible for the early gaseous fermentation.

The S-shaped curves obtained in cucumber extract with cultures of B. cereus (Fig. 5a), B. pumilus (Fig. 6b) and, to a lesser extent, B. subtilis (Fig. 5b) indicates an accumulation of peroxide in the culture medium. This is shown by the upward trend of the curves starting when the cultures were approximately 12 hours old.

The secondary drop in potential, coinciding with the appearance of growth in the medium, could be due to: (a) an adaptation of the organisms to the medium, (b) secondary growth of variants more capable of producing catalase in this medium, or (c) a latent formation of peroxidases.

Despite the fact that these three species grew comparatively well in cucumber extract, they produced but meager growth on the surface of

Fig. 5a,b,c and d. Time-potential curves of B. cereus, B. subtilis, B. mesentericus fuscus, and B. vulgatus in (a) cucumber extract and (b) nutrient broth.

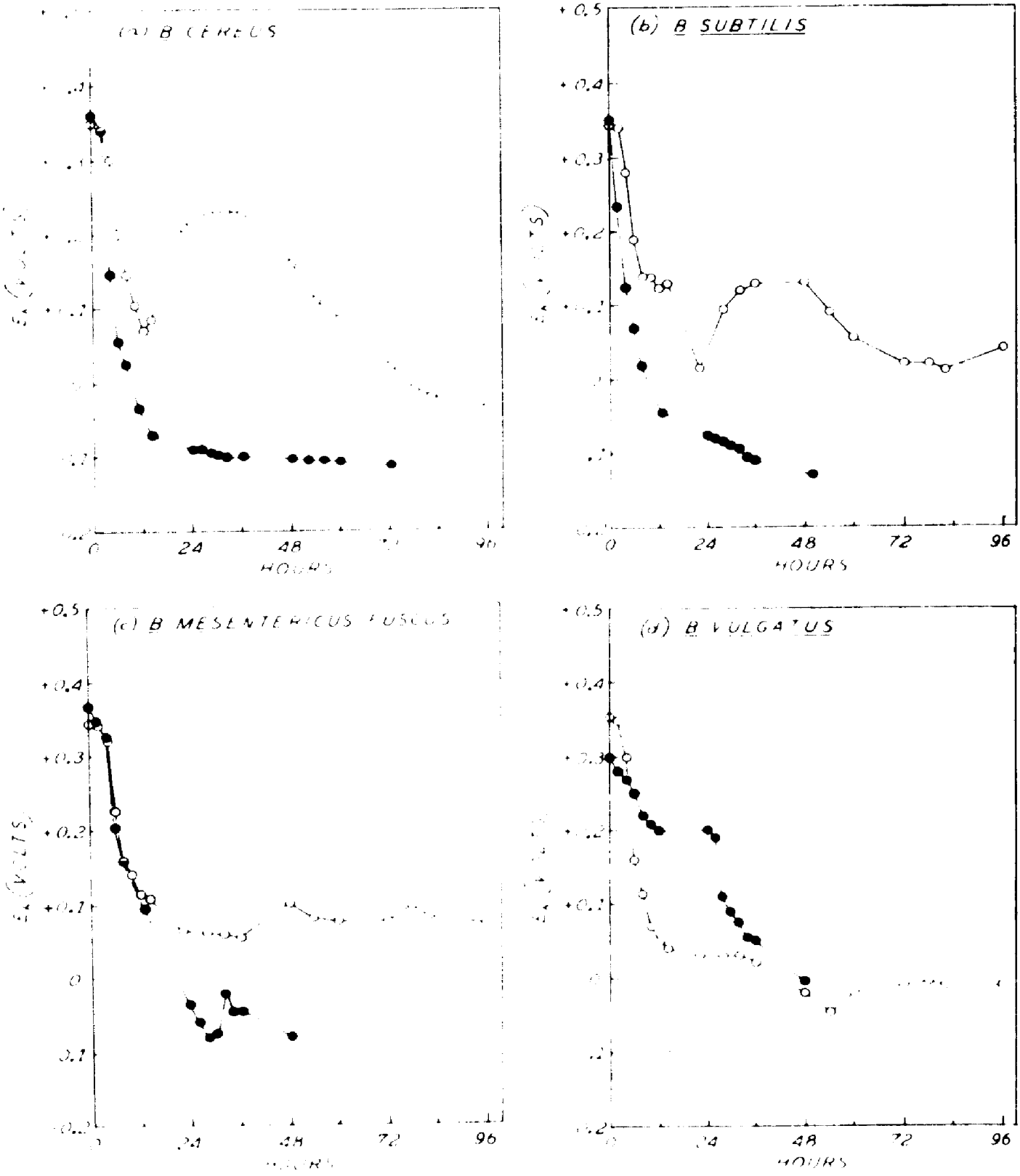
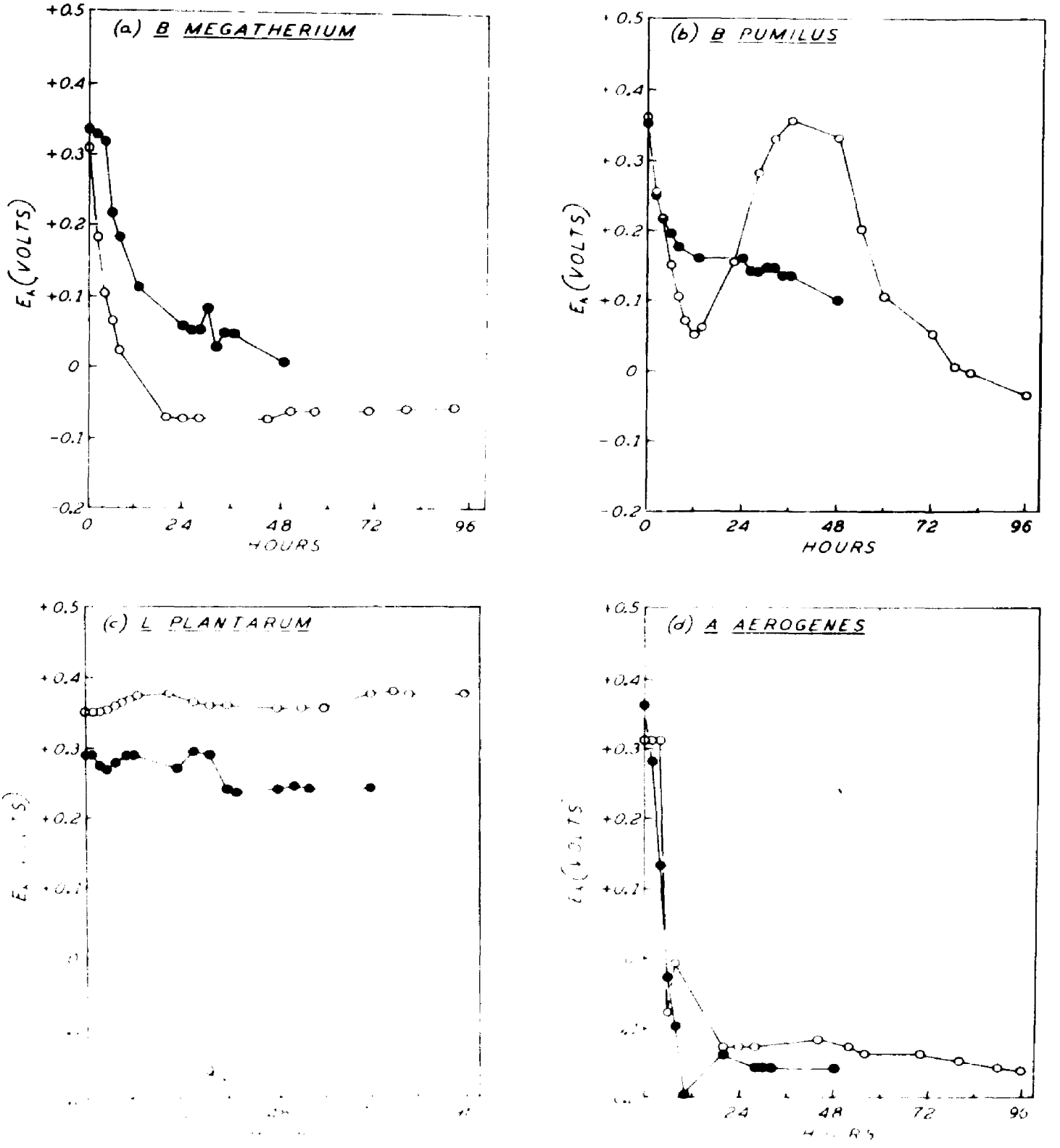


Fig. 6a,b,c and d. Time-potential curves of B. megatherium, B. pumilus, L. plantarum, and A. aerogenes in (a) cucumber extract and (b) nutrient broth.



cucumber extract agar slants. In order to determine whether this phenomenon could be correlated with catalase production, all six species of the sporogenic organisms were tested for their ability to elaborate catalase when grown on nutrient agar and on cucumber extract agar. All six species gave strongly positive tests for catalase when 1 per cent hydrogen peroxide was added to 24 hour agar slants. However, when similar tests were conducted using cucumber extract agar, B. cereus, B. pumilus, and B. subtilis gave negative tests for catalase. Aerobacter aerogenes, on the other hand, gave strongly positive tests on both media.

3. The effect of sodium chloride and lactic acid on the time-potential curves of aerobic sporogenic bacteria.

The influence of salt alone on the time-potential curves of the six species of sporogenic organisms was determined. Concentration of 2.5, 5.0, 7.5, and 10.0 per cent salt were obtained by adding aseptically 10 ml of a sterile double-strength salt solution to 10 ml of sterile double-strength proteose-peptone broth in the oxidation-reduction cells. This was done to minimize precipitation of the nitrogenous constituents of the medium at the high salt concentrations.

The cells were incubated 24 hours at 30°C to stabilize the potential and then inoculated with 1 ml of a 1-100 dilution of a 24 hour broth culture. Potential measurements were made at frequent intervals over a 48 hour period.

The combined effect of salt and lactic acid on the time-potential curve of Bacillus cereus was also determined. Salt concentrations of 0, 2.5, 5.0, 7.5, and 10.0 per cent were used in proteose-peptone broth which

had been adjusted to pH values of 4.0, 5.0, 6.0, and 7.0 with lactic acid.

Results and Discussion

From the data obtained (Fig. 7a,b,c and d and Fig. 8a and b), the principle effect of increasing the concentration of salt was a lengthening of the time required by the organism to reduce the potential of the medium to a level at which multiplication of the cells could take place. For example, in the case of Bacillus cereus (Fig. 7a), microscopic examination indicated a lag phase of four hours or more in those cultures containing no salt and 2.5 per cent salt, and over eight hours at 5.0 per cent salt. The cultures containing 7.5 per cent salt showed no indications of multiplication for more than 24 hours and those with 10 per cent did not grow in five days.

However, in all cases by the time the organisms had begun to multiply the potential of the medium had been reduced nearly three-tenths of a volt below its original level. These results suggest that at least a part of the bacteriostatic action of sodium chloride is due to its effect on the reducing enzymes of the organisms.

Although the degree of retardation of this potential drop with increasing salt concentrations varied considerably with the organisms used, there was only one exception to the general rule. Bacillus vulgatus (Fig. 7d) was able to establish a lower potential (-0.03 volts) in the presence of 7.5 per cent salt than when no salt was added to the medium. However, with 7.5 and 10 per cent salt, the sharp increases in potential after 36 and 24 hours respectively indicate an accumulation of

Fig. 7a,b,c and d. The effect of salt on the time-potential curves of aerobic sporogenic bacteria isolated from spoiled pickle brines.

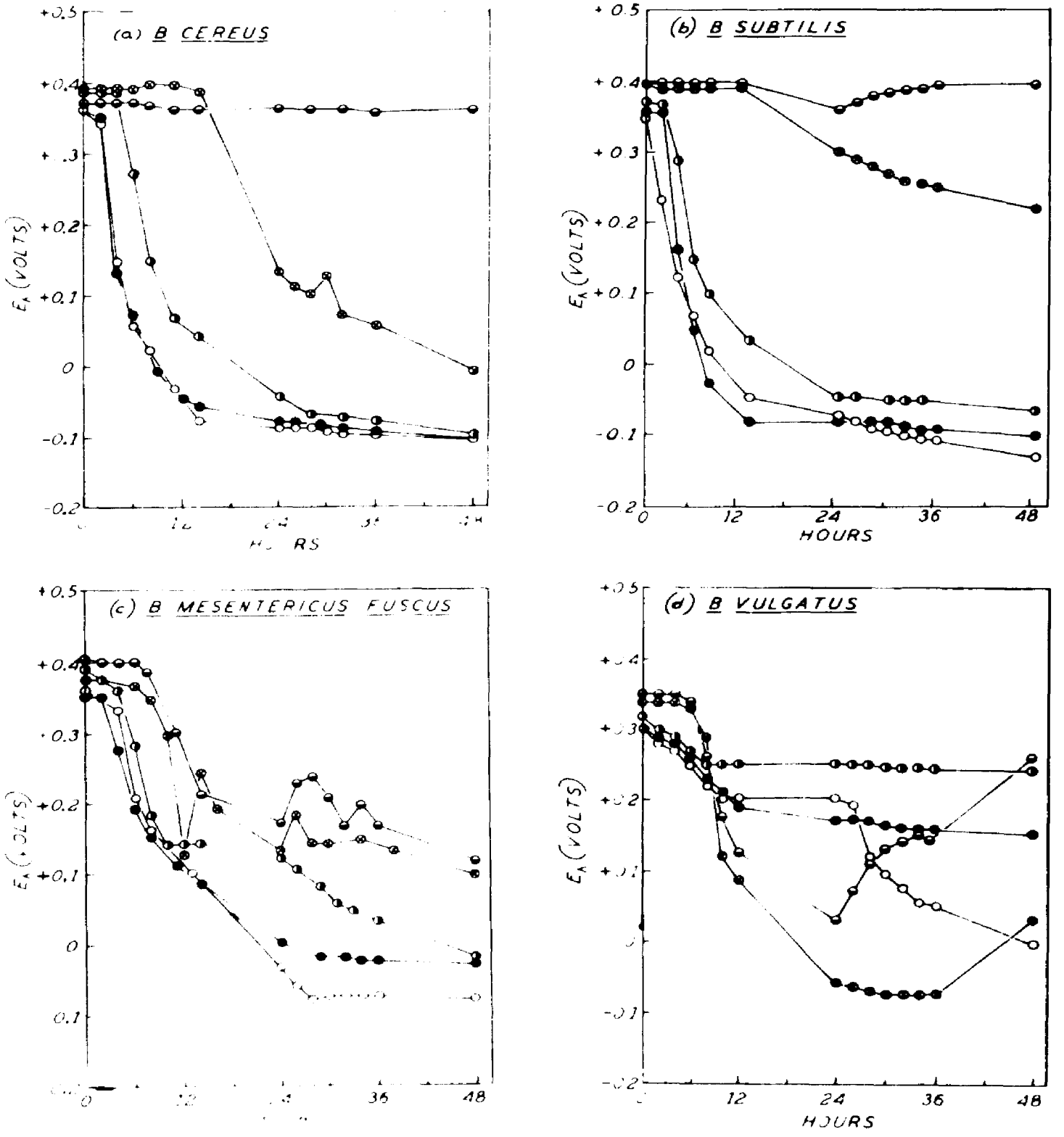
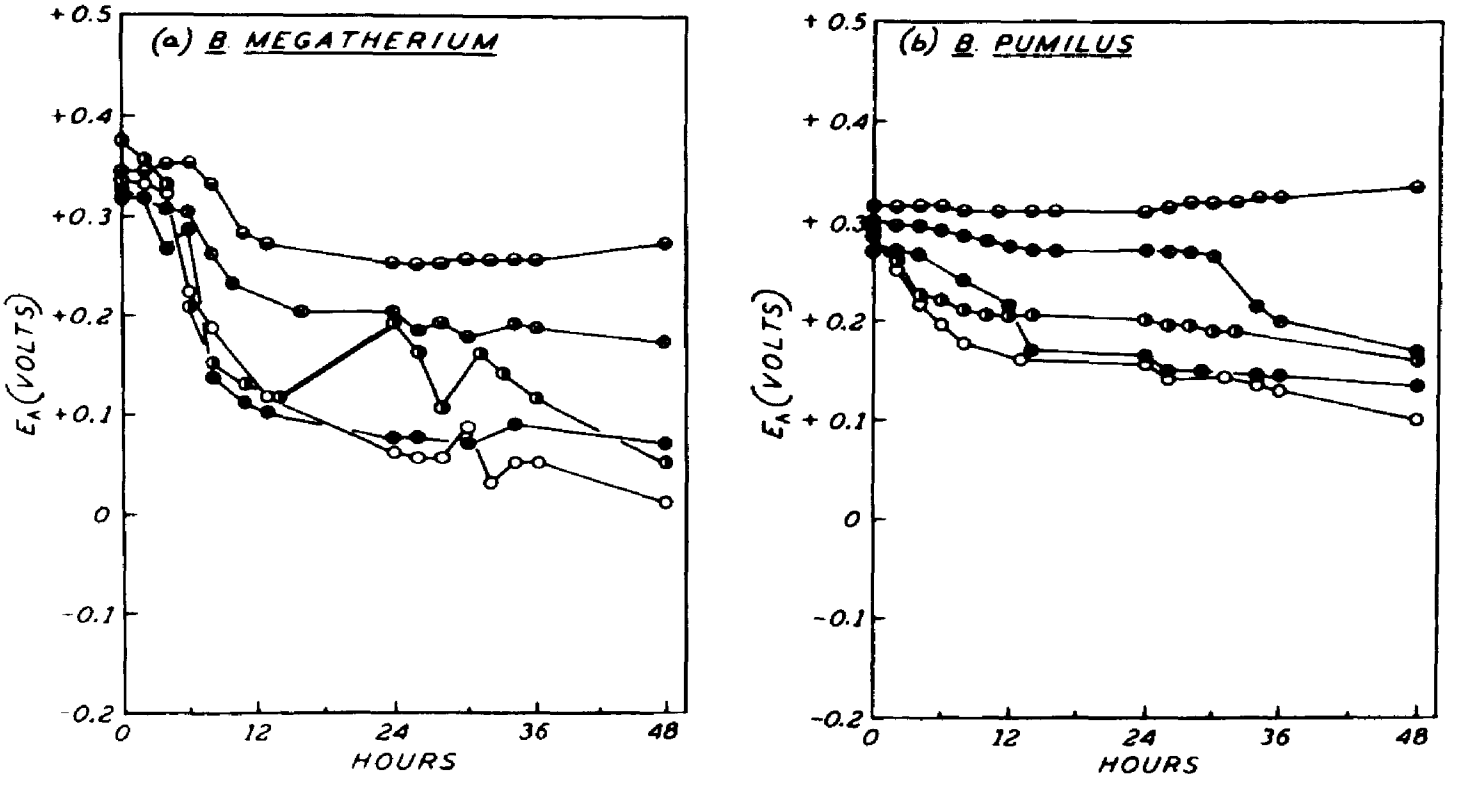


Fig. 8a and b. The effect of salt on the time-potential curves of aerobic sporogenic bacteria isolated from spoiled pickle brines.



peroxide in the medium. This presumably is due to impaired catalase activity in the higher concentrations of salt.

When lactic acid alone was added to the medium, the effect of increasing concentrations on the time-potential curve of B. cereus (Fig. 9) was very similar to the effects obtained by increasing the concentration of salt. When the initial pH of the medium was ajusted to values of 7.0 and 6.0 there was very little difference in the reducing activity of the organism. However, at pH 5.0 the potential drop was gradual and growth did not appear until after 48 hours. At pH 4.0 there was no decrease in potential or growth.

From the results obtained with combinations of lactic acid and salt (Fig. 10a,b,c, and d), it appears that the effects obtained when these compounds were added to the medium individually are additive when both are present. For example, B. cereus grew at pH 6.0 (Fig. 10b) when no salt was present and at a concentration of 7.5 per cent salt when the pH of the medium was 7.0 (Fig. 10a). However, this organism did not grow in the presence of 7.5 per cent salt when the medium was adjusted to pH 6.0 with lactic acid.

In general, these results indicate that the salt tolerance of this organism decreases with increasing concentrations of lactic acid.

4. The influence of the oxidation-reduction potential of the medium on the sensitivity of spore-formers to salt.

The purpose of this study was to determine whether the tolerance of spore-formers to salt could be changed by altering the potential of the medium in which they were grown. Bacillus cereus was selected for the

Fig. 9. The effect of lactic acid on the time-potential curves of B. cereus.

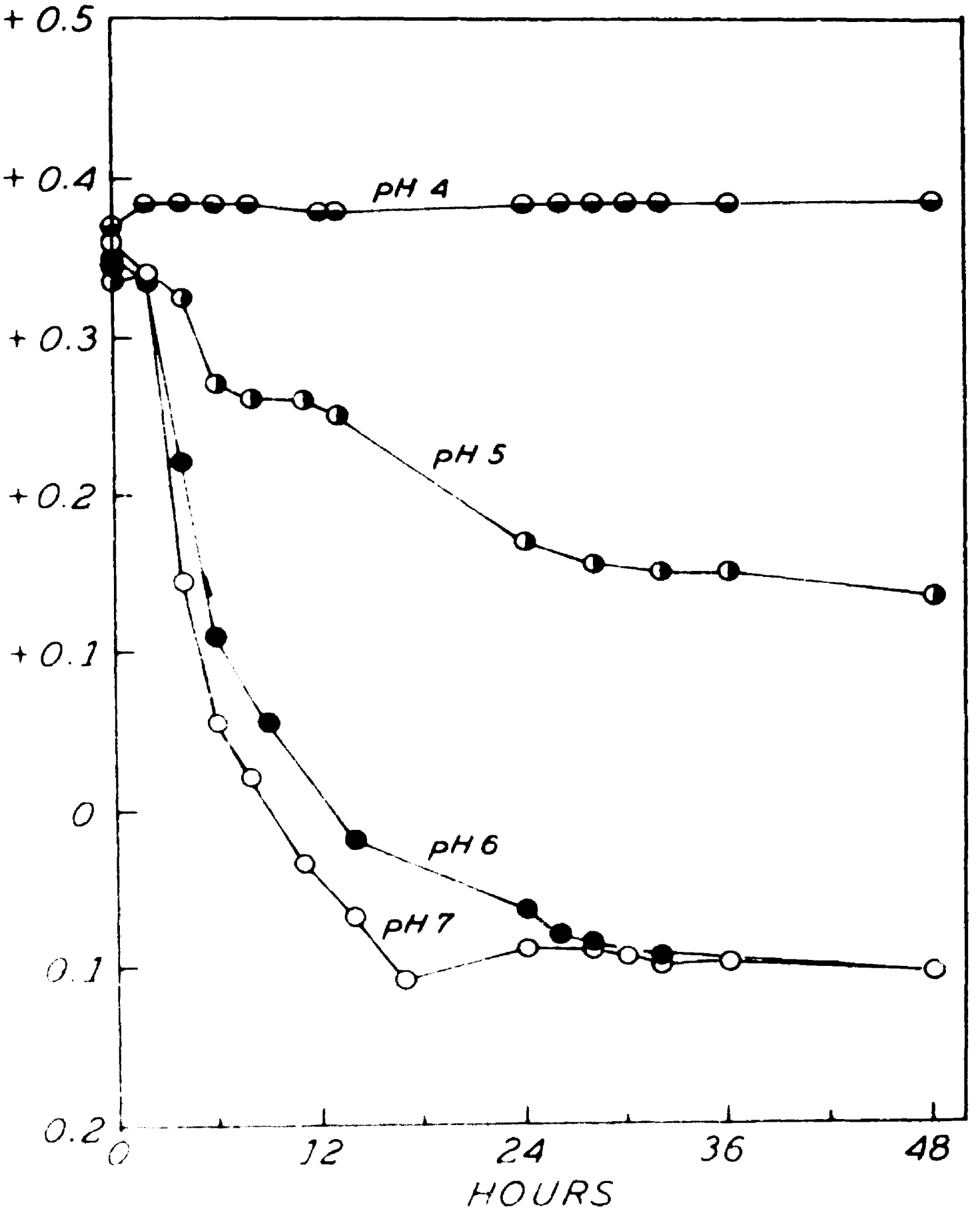
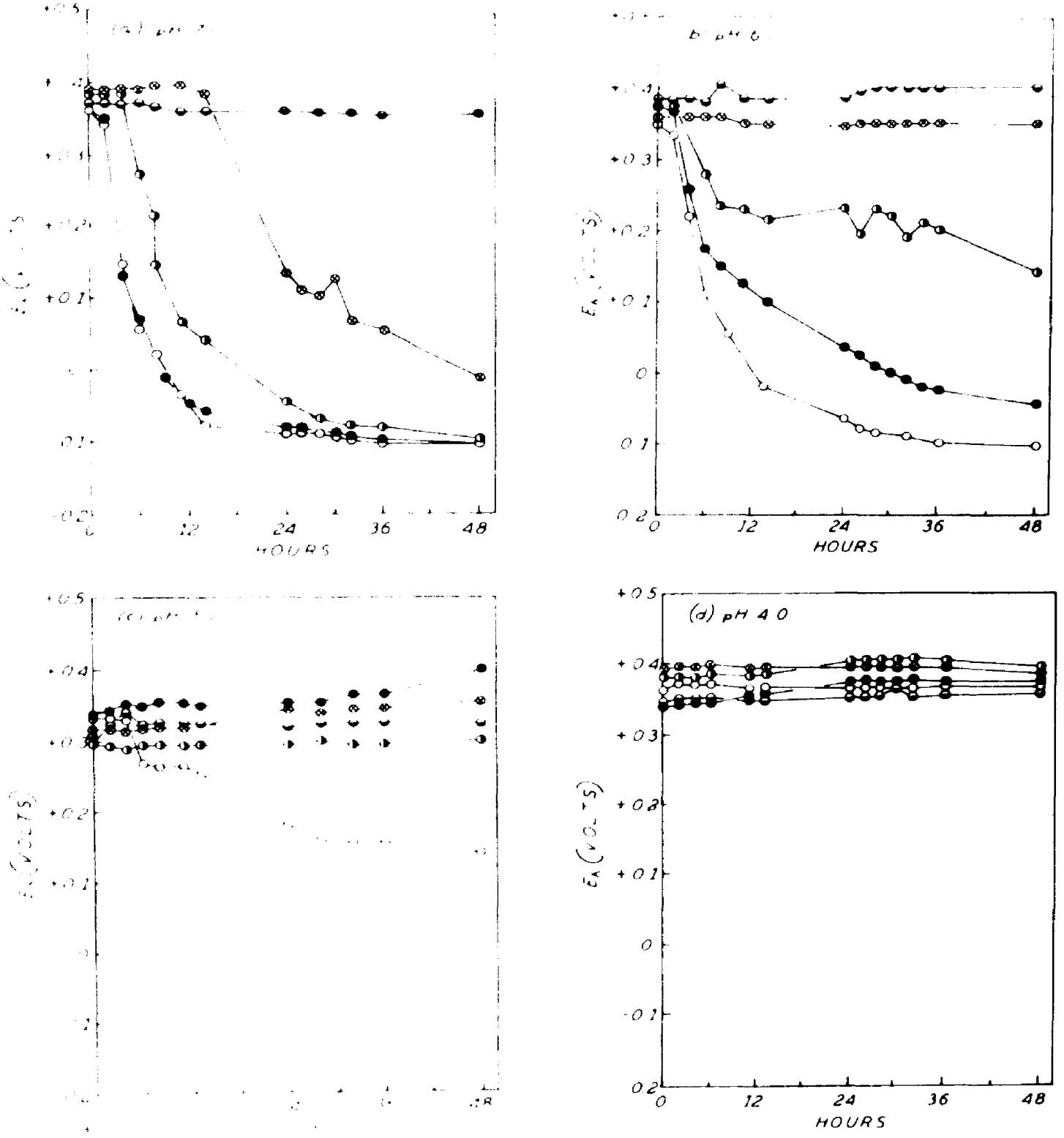


Fig. 10a,b,c and d. The combined effects of lactic acid and salt on the time-potential curves of B. cereus.



2.5 PERCENT SALT; 7.5 PER

PERCENT SALT.

preliminary studies since it was the predominant organism found in the brines. It was previously observed that the minimum potential attained by this organism in broth was approximately -0.100 volt and that active proliferation of the cells did not occur until the potential of the medium had been considerably reduced. For these reasons, attempts were made to reduce the potential to that limiting value. The use of reducing agents such as sodium sulfite did not prove successful since the concentrations required were too high and the potential attained with these substances was easily altered by jarring the tubes. Indigo carmine in a concentration of 1.3×10^{-4} molar only slightly reduced the medium as did reducing sugars such as glucose. However, a combination of the "poising" effect of the dye and the reducing action of 1 per cent glucose produced a satisfactory potential of -0.104 volts at 30°C.

In order to study the effect of potentials considerably lower than the limiting potential of the organism, a thioglycollate medium prepared by the Baltimore Biological Laboratory was used. At 30°C this medium had a potential of -0.204 volts.

The sensitivity of the six different species of bacilli to salt at high and low potentials were compared in both nutrient broth and cucumber extract.

Results and Discussion

The influence of the oxidation-reduction potential of the medium on the salt sensitivity of B. cereus is shown in Table 8. In ordinary nutrient broth (E_h = +0.28? volts) this organism grew in salt concentra-

Table 3. The effect of the oxidation-reduction potential of the medium on the sensitivity of B. cereus to sodium chloride.

Per cent NaCl	Medium A	Medium B	Medium C	Medium D	Medium E
0	+	+	+	+	+
1	+	+	+	+	+
2	+	+	+	+	+
3	+	+	+	+	+
4	+	+	+	+	+
5	+	+	+	+	+
6	+	+	+	+	+
7	+	+	+	+	-
8	+	+	+	+	-
9	+	+	+	+	-
10	-	-	+	+	-
11	-	-	+	+	-
12	-	-	+	+	-
13	-	-	+	+	-
14	-	-	+	+	-
15	-	-	-	-	-
16	-	-	-	-	-
17	-	-	-	-	-
18	-	-	-	-	-

Medium A = Nutrient broth (E_h = +0.288v)
Medium B = Nutrient broth + 1 per cent glucose (E_h = +0.208v)
Medium C = Nutrient broth + 1.8×10^{-4} indigo carmine (E_h = +0.278v)
Medium D = Nutrient broth + 1 per cent glucose + 1.8×10^{-4} molar indigo carmine (E_h = -0.104v)
Medium E = thioglycollate medium (E_h = -0.204v).

tions up to and including 9 per cent. Reduction of the potential of this medium to a value of +0.208 volts with 1 per cent glucose had no effect. However, when indigo carmine alone (E_h = +0.278 volts) or in combination with 1 per cent glucose (E_h = -0.104 volts) was added to the medium, it was found that the salt tolerance of this organism was increased to 14 per cent. When B. cereus was grown in the thioglycollate medium, growth was not attained in salt concentrations above 6 per cent. It was found that in those tubes where growth did occur, the organisms had raised the potential from the initial value of -0.204 volts to the limiting potential of that species, that is, approximately -0.100 volt.

Reduction of the potential of nutrient broth to -0.104 volts by the addition of indigo carmine and glucose tended to increase the salt tolerance of four of the six species tested (Table 9), but had no effect on the two remaining species. B. cereus (B-2) grew in 14 per cent salt at the lower potential in contrast to 9 per cent in ordinary nutrient broth. Similarly, B. mesentericus fuscus (C-1) was increased from 11 to 16 per cent, B. vulgatus (G-8) from 10 to 16 per cent, and B. megatherium (E-1) from 10 to 14 per cent. The salt tolerance of B. subtilis (F-1) and B. pumilus (G-7), on the other hand, was not affected by the lower potential. Both species grew up to and including 9 per cent salt at both high and low potentials.

When cucumber extract was used as the basal medium (Table 10), several significant differences were noted. B. cereus (B-2), B. subtilis (F-1), and B. pumilus (G-7) failed to grow above 3 per cent salt as com-

Table 9. The effect of oxidation-reduction potentials on the sensitivity of spore-formers to sodium chloride in nutrient broth.

Per cent NaCl	Type I (B-2)		Type II (f-1)		Type III (C-1)		Type IV (G-8)		Type V (E-1)		Type VI (G-7)	
	A	B	A	B	A	B	A	B	A	B	A	B
0	+	+	+	+	+	+	+	+	+	+	+	+
1	+	+	+	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+	+	+	+	+
7	+	+	+	+	+	+	+	+	+	+	+	+
8	+	+	+	+	+	+	+	+	+	+	+	+
9	+	+	+	+	+	+	+	+	+	+	+	+
10	-	+	-	-	+	+	+	+	+	+	-	-
11	-	+	-	-	+	+	-	+	-	+	-	-
12	-	+	-	-	-	+	-	+	-	+	-	-
13	-	+	-	-	-	+	-	+	-	+	-	-
14	-	+	-	-	-	+	-	+	-	+	-	-
15	-	-	-	-	-	+	-	+	-	-	-	-
16	-	-	-	-	-	+	-	+	-	-	-	-
17	-	-	-	-	-	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-	-	-	-	-	-

Medium A = Nutrient broth, (E_h = +0.288v)
Medium B = Nutrient broth + 1 per cent glucose + 1.8 X 10^{-4} molar indigo carmine, (E_h = -0.104v).

Table 10. The effect of oxidation-reduction potentials on the sensitivity of spore-formers to sodium chloride in cucumber extract.

Per cent NaCl	Type I (B-2)		Type II (F-1)		Type III (C-1)		Type IV (G-8)		Type V (E-1)		Type VI (G-7)	
	A	B	A	B	A	B	A	B	A	B	A	B
0	+	+	+	+	+	+	+	+	+	+	+	+
1	+	+	+	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+	+	+	+	+
4	-	-	-	-	+	+	+	+	+	+	-	-
5	-	-	-	-	+	+	+	+	+	+	-	-
6	-	-	-	-	+	+	+	+	+	+	-	-
7	-	-	-	-	+	+	+	+	+	+	-	-
8	-	-	-	-	+	+	+	+	+	+	-	-
9	-	-	-	-	+	+	+	+	-	+	-	-
10	-	-	-	-	+	+	-	+	-	+	-	-
11	-	-	-	-	+	+	-	+	-	+	-	-
12	-	-	-	-	-	+	-	+	-	+	-	-
13	-	-	-	-	-	+	-	+	-	-	-	-
14	-	-	-	-	-	+	-	+	-	-	-	-
15	-	-	-	-	-	+	-	-	-	-	-	-
16	-	-	-	-	-	+	-	-	-	-	-	-
17	-	-	-	-	-	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-	-	-	-	-	-

Medium A = Cucumber extract (E_h = +0.350)
Medium B = Cucumber extract + 1 per cent glucose + 1.8 X 10^{-4} molar indigo carmine, (E_h = -0.104v).

Table 9. The effect of oxidation-reduction potentials on the sensitivity of spore-formers to sodium chloride in nutrient broth.

Per cent NaCl	Type I (B-2)		Type II (f-1)		Type III (C-1)		Type IV (G-8)		Type V (E-1)		Type VI (G-7)	
	A	B	A	B	A	B	A	B	A	B	A	B
0	+	+	+	+	+	+	+	+	+	+	+	+
1	+	+	+	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+	+	+	+	+
7	+	+	+	+	+	+	+	+	+	+	+	+
8	+	+	+	+	+	+	+	+	+	+	+	+
9	+	+	+	+	+	+	+	+	+	+	+	+
10	-	+	-	-	+	+	+	+	+	+	-	-
11	-	+	-	-	+	+	-	+	-	+	-	-
12	-	+	-	-	-	+	-	+	-	+	-	-
13	-	+	-	-	-	+	-	+	-	+	-	-
14	-	+	-	-	-	+	-	+	-	+	-	-
15	-	-	-	-	-	+	-	+	-	-	-	-
16	-	-	-	-	-	+	-	+	-	-	-	-
17	-	-	-	-	-	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-	-	-	-	-	-

Medium A = Nutrient broth, (E_h = +0.288v)
Medium B = Nutrient broth + 1 per cent glucose + 1.8 X 10^{-4} molar indigo carmine, (E_h = -0.104v).

Table 10. The effect of oxidation-reduction potentials on the sensitivity of spore-formers to sodium chloride in cucumber extract.

Per cent NaCl	Type I (B-2)		Type II (F-1)		Type III (C-1)		Type IV (G-8)		Type V (E-1)		Type VI (G-7)	
	A	B	A	B	A	B	A	B	A	B	A	B
0	+	+	+	+	+	+	+	+	+	+	+	+
1	+	+	+	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+	+	+	+	+
4	-	-	-	-	+	+	+	+	+	+	-	-
5	-	-	-	-	+	+	+	+	+	+	-	-
6	-	-	-	-	+	+	+	+	+	+	-	-
7	-	-	-	-	+	+	+	+	+	+	-	-
8	-	-	-	-	+	+	+	+	+	+	-	-
9	-	-	-	-	+	+	+	+	-	+	-	-
10	-	-	-	-	+	+	-	+	-	+	-	-
11	-	-	-	-	+	+	-	+	-	+	-	-
12	-	-	-	-	-	+	-	+	-	+	-	-
13	-	-	-	-	-	+	-	+	-	-	-	-
14	-	-	-	-	-	+	-	+	-	-	-	-
15	-	-	-	-	-	+	-	-	-	-	-	-
16	-	-	-	-	-	+	-	-	-	-	-	-
17	-	-	-	-	-	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-	-	-	-	-	-

Medium A = Cucumber extract (E_h = +0.350)
Medium B = Cucumber extract + 1 per cent glucose + 1.8×10^{-4} molar indigo carmine, (E_h = -0.104v).

pared to 9 per cent in nutrient broth. Moreover, lowering the potential to -0.104 volts had no effect on the salt tolerance of these organisms.

B. megatherium (E-1) grew only up to 8 per cent salt at the higher potential but withstood 12 per cent salt at the reduced potential. The tolerance of B. vulgatus (G-8) and B. mesentericus fuscus (C-1) to the presence of salt in cucumber extract was likewise increased by reducing the potential. In the case of B. vulgatus, the concentration of salt could be increased from 9 to 14 per cent, while B. mesentericus fuscus grew in 16 per cent salt at the lower potential as compared to 12 per cent at the higher potential.

GENERAL DISCUSSION

The more the cucumber fermentation is studied the more complex it becomes. What at first appears to be a simple lactic acid fermentation has many variations depending upon a variety of conditions. Upon further consideration this is not altogether strange since with the cucumbers there is also introduced into the vats a large number of microorganisms from the soil. The microflora of the soil is extremely variegated including species of molds, yeasts, actinomycetes, and many bacteria. Generally speaking, the molds and actinomycetes do not find conditions to their liking and quickly disappear. It is the yeasts and bacteria which persist and which must be reckoned with.

Yeasts such as Debaromyces and Mycoderma abound on the surface and yeasts such as Torulaspora, Hansenula, and Brettanomyces abound in the depths of the brine. At the beginning of the fermentation two groups of bacteria are dominant, the aerobic sporogenic bacteria and the lactic acid bacteria. If only water were added to the cucumbers in the vats, they would all spoil and become a putrid mushy mess within 24 to 48 hours. However, the addition of salt in amounts as low as 2.5 to 3 per cent changes the picture entirely. The aerobic sporogenic bacteria which are responsible for the spoilage are suppressed and the lactic acid bacteria gain the upper-hand. As soon as this latter group produces lactic acid the conditions for growth of the sporeforming spoilage bacteria become increasingly unfavorable until they are entirely suppressed or disappear. This is what usually happens in the normal fermentation, otherwise the pickle salter and manufacturer would have to go out of business.

However, there is always the occasional tank that spoils and in some years under abnormal conditions of temperature and rainfall there may be several tanks that spoil for no apparent reason. In checking back over the records there is absolutely nothing to indicate why any of the tanks should have spoiled since they were all treated similarly and were filled with cucumbers grown in the same region. It was to study the possibilities under which the spoilage bacteria (aerobic sporogenic bacteria) could grow and multiply to the point where they could elaborate sufficient enzymes to cause spoilage that this work was undertaken.

In this connection there appeared several possibilities. First, that since bacteriophage was known to lyse or destroy bacteria in other commercial fermentations such as the lactic acid fermentation in the cheese industry and the acetone-butyl alcohol fermentation, there was the possibility that bacteriophage might destroy or suppress the lactic acid organisms to a point where the aerobic sporogenic spoilage bacteria of the B. mesentericus fuscus or B. vulgatus types might grow sufficiently so as to produce enough pectolytic enzymes to soften the cucumbers within a few months time. Since enzymes act as catalysts, small amounts of the pectolytic enzymes acting over a period of several months could easily produce softening. It is seldom that a tank of pickles spoils within a short time after it has been salted. Several months are usually required for the softening to occur.

In several cases of spoilage of genuine dills the evidence pointed very strongly to this possibility of spoilage by bacteriophage. In one

case in Canada where several hundred barrels of genuine dills spoiled, there was strong indication that bacteriophage was the cause of the spoilage, but due to the age and acidity of the brines this could not be definitely proven. Several years ago another pickle plant in Michigan had considerable trouble with genuine dill pickles when they changed from a well water to lake water which was contaminated. In this study bacteriophage capable of lysing cultures of Lactobacillus plantarum isolated from cucumber fermentations was found in the soil of a cucumber patch. This indicates that the phage is there and needs only the proper conditions to develop.

The second possibility to account for spoilage is that of antibiotic substances being elaborated by the aerobic sporogenic spoilage bacteria in sufficient quantities to suppress or kill off the lactic acid bacteria. In this study six species of spoilage bacteria were isolated from pickle brines and grown in several media until they had had ample time to produce an antibiotic substance. The filtrates were then tested for their antibiotic properties against Leuconostoc mesenteroides, six species of lactobacilli isolated from non-pickle sources and 10 strains lactobacilli isolated from pickle brines. The results were clear-cut and positive. The filtrates of two organisms, B. mesentericus fuscus and B. vulgatus, when grown in a cucumber infusion, showed strong inhibiting action against all strains of lactobacilli isolated from pickle brines. It is interesting to note how much more active they were in this medium than in the other media. Incidentally, these were the only two organisms which had the

ability of producing enzymes capable of softening cucumbers. It would, therefore, seem that they were capable of acting in two ways. First, they can elaborate antibiotic substances capable of inhibiting the growth of lactobacilli and at the same time produce an enzyme capable of softening the pickle.

The third possibility which was considered was that of establishing conditions in the pickle brine favorable for growth of the spoilage bacteria because unless these bacteria can grow they cannot produce either antibiotics or pectolytic enzymes. To explore this possibility, oxidation-reduction potentials were run on actively fermenting pickle brines as well as on the different organisms isolated from these brines. It was found that fermenting cucumber brines were actively reducing during the first few days. It was also found that the six different species of spoilage bacteria had the ability to substantially reduce the potential of the medium in which they grew while Lactobacillus plantarum, the organism mainly responsible for the pickle fermentation, reduced the potential very little if at all. The ability of the spoilage bacteria to reduce the oxidation-reduction potential is significant since it shows their ability to establish conditions favorable for their growth.

In this connection it is interesting to note the influence which the oxidation-reduction potential of the medium exerts on the amount of salt which the spoilage organisms can tolerate. It was clearly demonstrated that by artifically reducing the potential to a value approximating the lowest potential established by the organisms, most of the

species could be grown in considerably higher concentrations of salt. This would indicate that at least a part of the bacteriostatic action of salt is due to its effect on the reducing enzymes of the organisms, a fact which would explain the greater tolerance of lactobacilli to salt since these organisms do not require a highly reduced medium for proliferation of the cells.

Even more significant were the studies of the influence of salt and lactic acid on the time-potential curves of the spoilage bacteria. The principle effect of increasing the quantity of salt in the medium was to lengthen the time required by the organisms to reduce the potential of the medium to a level at which multiplication of the bacterial cells could take place. In other words, the addition of salt increased the lag phase of the organism. For example, when B. cereus was grown in media containing either no salt or 2.5 per cent salt, there was a lag phase of approximately four hours before the cells started to proliferate. However, when the concentration of salt was increased to 5 per cent there was a lag phase of approximately eight hours. Thus, in this particular instance a twofold increase in salt resulted in a doubling of the lag phase and at higher salt concentrations the lag phase was greatly lengthened. For example, in the presence of 7.5 per cent salt, the cells did not begin to multiply for more than 24 hours and had not grown at the end of five days in the presence of 10 per cent salt.

When lactic acid was added to the medium in the presence of different concentrations of salt, the effect was additive. For example, B. cereus grew at pH 6.0 without salt and at 7.5 per cent salt at pH 7.0,

but would not grow at pH 6.0 and 7.5 per cent salt. This shows that the salt tolerance of this organism decreases with increasing amounts of acid. These results indicate very clearly the role that sodium chloride and lactic acid play in the cucumber fermentation.

SUMMARY

Bacteriophage races which lysed Lactobacillus plantarum cultures was isolated from the soil where cucumbers had grown but not from water or from genuine dill pickle brines in which spoilage had occurred six to eight months previously.

Aerobic sporogenic bacteria commonly found in the soil and in fermenting cucumber vats are capable of producing antibiotic substances which greatly inhibit the growth of Lactobacillus plantarum, the organism chiefly responsible for the fermentation of cucumbers.

Bacillus mesentericus fuscus and Bacillus vulgatus, the two species capable of producing substances antagonistic to Lactobacillus plantarum in a cucumber infusion, are also able to elaborate pectin-hydrolyzing enzymes which are responsible for the softening of cucumber pickles.

All six species of aerobic sporogenic bacteria isolated from spoiled pickle brines reduced the oxidation-reduction potential of the medium to a considerable degree while the lactobacilli had little or no effect on the E_h.

Increasing the sodium chloride content of the medium tended to increase the time required by the organisms to reduce the potential of the medium to a level at which multiplication of the cells could take place.

When lactic acid was added to the medium the effect was similar to that obtained when salt alone was used but when lactic acid and sodium

chloride were added conjointly these effects were additive, thus indicating that the salt tolerance of these organisms decreases with increasing concentrations of lactic acid.

Most of the spoilage organisms could be induced to grow in significantly higher concentrations of salt if the oxidation-reduction potential of the medium was artificially reduced to the minimum level established by the organisms during normal growth.

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