A NEW TYPE OF GASEOUS FERMENTATION OCCURRING DURING THE SALTING OF CUCUMBERS

by

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INTRODUCTION

Approximately 6,000,000 bushels* of pickling cucumbers are raised annually in the United States, representing a cash income of about $3,600,000 to the growers. Since only a small acreage, usually one to two acres, is planted per grower it is estimated that approximately 60,000 farmers are benefited by the pickling industry.

The commercial salting procedure** in brief is as follows: Large wooden vats of 400 to 1000 bu. capacity are filled with green cucumbers, fitted with a false head and 40 per cent saturated salt brine added so as to come a few inches above the heads. The initial salt concentration is raised according to a set schedule so that the salt concentration reaches 50 to 70 per cent saturation by the end of the sixth week. An acid fermentation commences shortly after the cucumbers are brined and continues for from two to six weeks.

The initial inoculum of microorganisms for the fermentation comes from the cucumbers and from adhering particles of soil. The salt-tolerant organisms utilize as their nutritive material the soluble constituents that diffuse into the brine as the result of the action of salt on the cucumber tissue. The action of the microorganisms on the fermentable material in the brine brings about the production of various compounds (chiefly lactic acid, also acetic acid and alcohol) as well as the evolution of considerable quantities of gas.

At the completion of the curing process (about 3 months), the cucumbers have changed from the green, opaque, air-filled, bouyant fruit to olive colored, translucent, air-free salt stock.

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* Agricultural Statistics, U.S.D.A., Washington, D. C. (1939).
** For 40 per cent saturated brine treatment.

It is clearly evident that the curing process is one of complex bacteriological, chemical and physical changes. Any one of these changes constitutes adequate basis for considerable fundamental investigation in an attempt to gain a better understanding of the principles involved during curing. Such studies would go a long way in placing the pickling industry in the group of scientifically controlled fermentations.

For the past 40 years research has dealt principally with the bacteriology of the lactic acid fermentation, with a very small proportion of the work being carried out under plant conditions. Little or no attention has been given to the fact that probably fermentations, due to microorganisms other than the lactic acid bacteria, existed and could contribute to the general fermentation. That such is the case is evidenced by the fact that the true yeast fermentation, associated with the general cucumber fermentation, was not recognized by workers in this field until as late as 1940 (12). Similarly, combined bacteriological and chemical investigations upon cucumber fermentations at certain salt concentrations point toward the inclusion of still another phase in the fermentation proper. The latter phase is brought about by a group of gas-producing salt-tolerant microorganisms and can be detected most easily by the evolution of gas which is composed of approximately equal parts of carbon dioxide and hydrogen.

The isolation, identification and physiological studies of this group of organisms as well as the observations dealing with their typical fermentation under plant conditions constitute the basis for this study.

HISTORICAL REVIEW

The literature on cucumber fermentations will be reviewed chiefly with respect to the bacteriological aspects of the following topics: Lactic acid fermentation; yeast fermentation and the hydrogen fermentation.

Until recently, bacteriological investigations on brine cucumber fermentations (for salt stock) have dealt principally with that phase of the fermentation resulting chiefly in the production of lactic acid. As early as 1899 Aderhold (1) studied the acid fermentation in dill brines and found that it was fostered by anaerobic conditions and that such conditions resulted in the production of more acid. In 1909 Kossowicz (24) confirmed Aderhold's work and also studied the flora of the active fermentation in dill brines. In addition experiments on the use of pure cultures for starters for dill pickle fermentations were carried out. The observations of these workers, while conducted on dill brines, are particularly interesting and can be considered of fundamental significance.

Lactic Acid Fermentation.- Prior to 1913 there was little or no work published in this country on cucumber fermentations. In 1913, Rahn's (29) bacteriological study of cucumber fermentations revealed that actively fermenting brine contained as many as 200,000,000 acid-forming bacteria per cc. and the acidity of the brine at the end of active fermentation reached 0.6 to 1.2 per cent lactic acid.

Brown (8), in 1916, in an abstract of an unpublished article, made a series of observations covering many aspects of cucumber fermentations. In reference to the production of acid, he describes the

responsible microorganisms as short rods or cocci, arranged chiefly in chains and being facultative with respect to oxygen requirements. He further observed that the ratio of lactic to acetic acid was 2 to 1 and that other acids, propionic, butyric and benzoic occurred in traces.

During the years 1919, 1920, 1922, Le Fevre (25, 26, 28) reported his observations on a series of experiments. He pointed out that cucumbers, as they came in from the field carried numerous bacteria, the principal groups being; "lacto-bacilli", aerobic spore-formers, gas-formers, yeasts and molds. The "lacto-bacilli" were claimed to be the most tolerant of salt and were considered the most significant in bringing about acid production. Thirty degrees C. was found to be the optimum temperature for the "lacto-bacilli". The addition of fermentable sugar was reported to be advantageous in bringing about a quicker onset of the fermentation and resulted in a higher degree of acidity. Le Fevre also pointed out that a higher acid content developed in the higher as compared to the lower salt content brines. Campbell (9) reported (a) that the microorganisms responsible for the souring of milk were concerned with the cucumber fermentation; (b) that the lactic group of organisms should be supplied with oxygen; (c) that yeasts played a desirable role in the fermentation. Le Fevre (27) took issue with Campbell (9) because of the latter's misconception of the bacteriological changes during cucumber fermentations. A careful inspection of Campbell's report (which included no data) shows that he was undoubtedly confusing true yeasts with Mycoderma (false yeasts) originating from the heavy surface scum.

Tanner (36), 1926, in a discussion of the curing process, claimed that the brine acidity resulted mainly from volatile acids rather than lactic acid. He was of the opinion that while lactic acid was generally mentioned as the predominating acid, this was usually done without adequate chemical examination. The curing process was said to be chiefly an acid-gas fermentation.

Fabian (13), in 1930, presented a general discussion of the considerations of pickle manufacture and referred principally to the observations of Le Fevre which have been discussed previously. Fabian also pointed out that the production of cucumber pickles was a scientific process and a thorough knowledge of the fundamentals was essential for successful manufacture. The influence of salt was stressed both from the osmotic effect for withdrawing food material for bacteria and for its effect in encouraging lactic acid bacteria to the exclusion of putrefactive bacteria. During the same year Joslyn (23) presented a general article in relation to pickle manufacture. His discussion of the lactic fermentation was the same as that of earlier authors.

It is well to point out that the work on the acid fermentation of brined cucumbers up to about 1930 consisted for the greater part of general discussions and opinions as viewed by the various authors and directed toward the industry with very little bacteriological or chemical data included as supporting evidence for the conclusions that were made. However, the work definitely presents many interesting and valuable contributions to the general understanding of the bacteriological processes of brine cucumber fermentations. Tanner and Eagle (37) in an excellent general review of the literature through 1926 more or less

summed up the situation when they stated, "Despite the fact that this fermentation is an old one, much research may yet be done and must be done before it may be considered a controlled fermentation industry. There is a great need for combined microbiological and chemical investigations." They might also have added that there was a great need for investigations to be carried out under conditions which could be considered typical of the industry, a point which up until 1930 had not been given serious consideration.

Although a routine differential plating medium suitable for detecting acid-forming organisms (weak and strong and peptonizing bacteria) was reported and described in great detail by Ayers and Mudge (4) in 1920, no adaption was made of this medium to cucumber fermentations for a full 12 years. Fabian and co-workers (14), in 1932, in perhaps the first systematic study of the microflora and chemical end-products of cucumber fermentations, used this medium to advantage in determining the populations of acid-forming organisms occurring in brines of initial strength of 8 and 10.6 per cent salt concentration. In addition to the study of the effect of acids, bases and salts on the fermentation, chemical determinations were carried out during the fermentations with respect to total acids, volatile acids, alcohol and reducing sugars. The results of the investigations, in general, showed that the flora of the fermentation with respect to acid-forming organisms consisted chiefly of weak acid-formers and that there was a greater number of acid-formers in the weaker brines, and they reached a maximum sooner than in the stronger brines. The chemical analyses showed that there were greater amounts of non-volatile acids, volatile acids and alcohol

in the weaker brines as compared to the stronger brines. In 1935, Vahlteick, Haurand and Perry (38) attempted one of the first bacteriological studies conducted under commercial conditions. They studied the microflora during the curing of cucumbers salted at a brine concentration of 10 per cent. The data from their limited observations on two vats led them to believe that the following groups of microorganisms were present: High acid producers of the Lactobacillus type; low acid producers of the Leuconostoc type; round yeasts; ellipsoidal yeasts (scum yeasts); spore-forming bacteria; and unclassified. The formation of acid during fermentation was attributed to acid-producing organisms identified as Lactobacillus cucumeris and to two species of the Leuconostoc genus. Wustenfeld and Kreip (42), 1933, divided the lactic acid bacteria into the following genera; Bacillus, Streptococcus, and Pedococcus. Furthermore, they stressed the desirability of the addition of pure cultures as starters subsequent to thorough washing of the cucumbers at the time of salting. Jones (21), in 1940, carried out extensive studies over a three year period at a commercial pickling plant on the salting of cucumbers in barrels. Fermentations in initial brine concentrations of 20, 30, 40, 60 and 80 per cent saturation brought about increased acid production in the reverse order named. In addition, he proved that higher acidity resulted from the fermentations (at any given salt concentration) when cucumbers of the smaller sizes were employed. The reverse was true when the larger sizes of cucumbers were used.

It is of interest to consider the effect of added sugar on the lactic acid fermentation. Numerous investigators (10, 15, 16, 22, 26,

42) have suggested that beneficial results may be obtained by the addition of sugar to cucumber fermentations and that these benefits may be reflected in an accelerated rate of acid production or in an increased production of lactic acid. Also, it has been reported that both acceleration and increased acid production can be obtained. However, in some cases it would seem that not a sufficient amount of work was carried out under conditions typical of the industry to justify the extensive conclusions presented. In contrast to those reporting beneficial results, Vahlteick, Haurand and Perry (38) were unable to stimulate the acid fermentation by the addition of sugar to some of their commercial vats. Veldhuis and co-workers (40) were unable to demonstrate a significant increase in brine acidity in the resulting fermentations to which sugar was added over that of the control lots. In their experiments sugar was added to different fermentations as follows: At the start, after 10 days, and in small amounts at short intervals during the fermentation. These investigators emphasized the importance of conducting well replicated experiments with adequate controls before conclusions might be drawn safely concerning the influence of addition of sugar to brines at the start or during fermentation.

Yeast Fermentation.- Although true yeasts have been mentioned in the literature in connection with cucumber fermentations (Kossowicz (24); Hasbrouck (19); Riley (31); Brown (8); Le Fevre (27,28); Joslyn (23); Vahlteick et al. (38); Campbell (10)), no systematic study as to their populations in brine was recorded until very recently. In 1940, Etchells (12) showed that a part of the typical fermentation of cucumbers was brought about by yeasts. Yeast fermentations were found in brine treat-

ments of 20, 30, 40 and 60 per cent saturation with respect to salt. It was shown that there was no direct correlation between brine concentration and maximum number of yeasts present. In an earlier report, Veldhuis and Etchells (39) carried out probably the first systematic study of gases evolved during the fermentation of cucumbers salted under what could be called commercial conditions at various salt concentrations (20 to 80 per cent saturation). A definite correlation was reported between carbon dioxide production and the presence of typical yeast fermentations. Other interesting points of the above work, particularly that dealing with the presence of hydrogen in the evolved gases, will be discussed under the following topic heading.

Hydrogen Fermentation.- A thorough review of the literature on cucumber fermentations up until 1939 reveals no study, either chemical or bacteriological, dealing with that phase of the fermentation during which a mixture of carbon dioxide and hydrogen is evolved. However, it may be noted that hydrogen was mentioned casually upon at least two occasions when it was claimed to be a product of the undesirable microorganisms at the start of fermentations (8, 27). Upon another occasion it was claimed present among numerous other by-products (23). The first conclusive evidence that showed hydrogen represented a portion of the evolved gas from certain cucumber fermentations was presented by Veldhuis and Etchells (39) in 1939. In this work it was shown that hydrogen was produced in considerable quantities in all fermentations observed in 60 per cent saturation brines. Examination of gas from hollow cucumbers or "bloaters" from 60 per cent saturation brines showed it to have about the same composition with respect to carbon dioxide and hydrogen as the

corresponding surface gas. The bacteriological investigations reported were of a limited nature. It was pointed out however, that an organism could be isolated from this typical hydrogen fermentation which was capable of producing a relatively high percentage of hydrogen.

ISOLATION STUDIES

From the historical review it is evident that, prior to 1939, no study had appeared dealing with either the chemical or bacteriological aspects of that phase of cucumber fermentations, during which hydrogen constituted a portion of the evolved gases. The original study (39), which dealt principally with analyses of the evolved gases from cucumber fermentations, showed that hydrogen represented a portion of the evolved gases, especially those carried out at 60° salometer brines*. The above report also mentioned that an organism could be isolated which was associated with the production of hydrogen. It is of interest here to review somewhat completely the bacteriological problems involved during this work inasmuch as it was through continuation of the initial observations that the stock cultures were subsequently isolated and used to constitute a portion of the present work.

The first observations (1937 season) on the gases evolved from cucumber fermentations were undertaken at the field laboratory located at the plant of the Chas. F. Cates Co., Faison, N. C. The various salting procedures were carried out in vats of approximately 85 bu. capacity. Additional observations on commercial fermentations were made at the Mt. Olive Pickle Company, located at Mt. Olive, N. C.

During the study of the composition of the evolved gases, it was found that, in addition to carbon dioxide, hydrogen was present in the gases from some of the fermentations. During this period, the bubbles forming on the surface of the vat brine would explode with a distinct report when lighted with a match.

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* Per cent saturation with respect to salt.

Numerous attempts were made during the 1937 season to isolate the causative microorganisms from the fermentations evolving hydrogen by the ordinary plating methods available, but all proved futile.

During the following season (1938), a vat salted at 40° salometer and located at Mt. Olive, a short distance from the field laboratory, was found to have a vigorous gaseous fermentation two days after having been put down and the gas analyses showed a relatively high percentage (37.0 per cent) of hydrogen present. Since this fermentation was thought to present an ideal opportunity for bacteriological study, plate counts were made at this time using nutritive caseinate agar (Difco) and tartaric acid agar. These media revealed the following groups of organisms present per cc. of brine: 22 million tiny acid-formers, 200 thousand peptonizing bacteria of the soil type, and 200 yeasts. Numerous isolations of the predominating organisms present were made and tested in fermentation tubes, using cucumber juice broth as the liquid medium, but no visible gas was produced by any of the resulting fermentations. Hence, it was assumed that the organisms predominating in the fermentation, as evidenced by the plate counts, were not responsible for the hydrogen evolution. Also, it was obvious that the routine aerobic plating methods that had been devised for following the brine fermentations with respect to acid-formers, peptonizers and yeasts were not suitable for isolation of the hydrogen-producing organisms. Whether the difficulty was due to growth requirements of the organisms in question or due to interference by other groups present in the brine, remained to be determined.

Since the fermentation in the vat described above was relatively

short, and also, since it was not on the premises where the laboratory was located, no further observations could be carried out. However, later on, in other vats, a more intensive effort was made to cultivate the microorganisms responsible for the hydrogen evolution. This involved numerous platings and the employment of various types of solid media. Finally, it was demonstrated that by use of strict anaerobic conditions*, a suitable differentiation of the brine microflora could be obtained and the organisms revealed in sufficient numbers so that typical colonies could be picked and studied. Once the colonial characteristics were established, numerous strains were isolated by this method.

Since the organisms proved to be facultative anaerobes, it was obvious that other cultural difficulties prevented reasonably easy isolations by ordinary aerobic cultural methods. Later studies showed that the plate counts indicated that the hydrogen-producing organisms were present in the brines in relatively low numbers as compared to the total numbers of microorganisms present. Also, it was found that the use of a culture medium suitable for the growth of the acid-forming bacteria, resulted in a low pH of the medium, which was inhibitive to the hydrogen group. Furthermore, the employment of a medium which excluded the acid-forming bacteria, encouraged the spreading, aerobic spore-forming types, making the plates valueless for numerical counts or isolation of the organisms producing hydrogen. The latter difficulty was particularly true under laboratory growth conditions prevailing at

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* Large desiccators used for anaerobic jars, freed of oxygen by the addition of excess of alkaline pyrogallol mixture followed by drawing a vacuum of 15 to 20 inches of mercury. (See Fig. 1.)

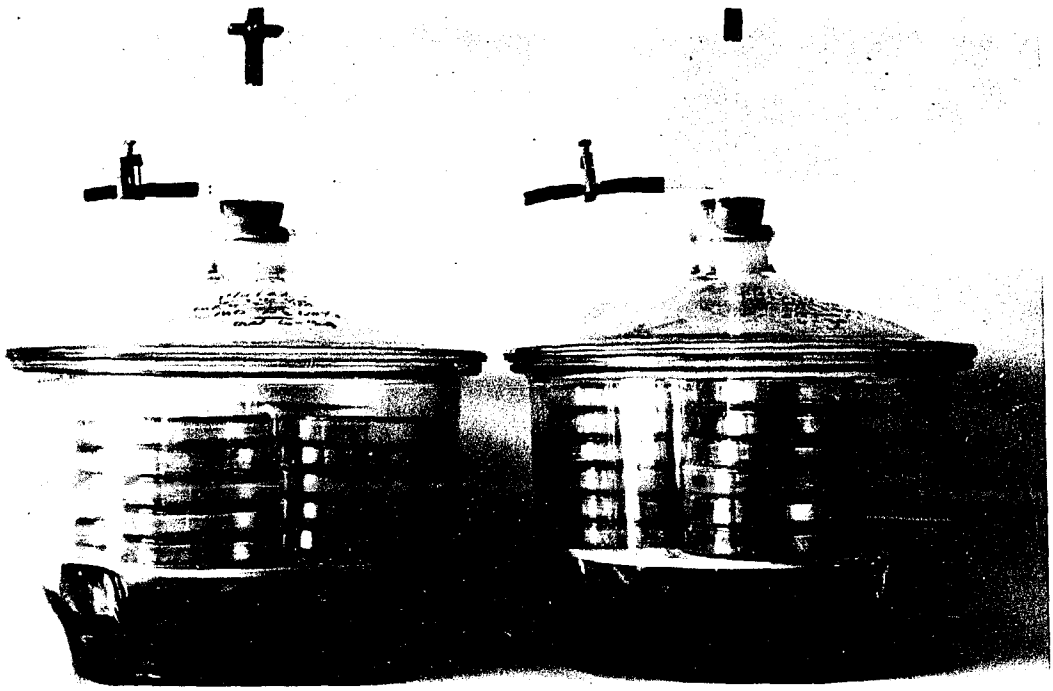

Fig. 1. Anaerobic culture jars used in the isolation studies. Desiccator, vacuum type of Pyrex glass with tubulated cover, inside diameter of 250 mm., capacity about 24 Petri dishes. Opening in cover supplied with a two hole, No. 8 rubber stopper fitted with two pieces of ordinary glass tubing; a straight piece running to the bottom of the jar, used for the addition of the alkali and a short, right angle piece for drawing and releasing vacuum. Both tubes supplied with a short piece of vacuum rubber tubing and a screw clamp. Solutions in the order used: (a) 400 cc. of pyrogallol solution, (142 g. per liter) placed in the bottom of the jar; (b) 400 cc. of KOH solution (222 g. per liter) added through the straight tube.

the field station during the hot, humid weather which is typical during the summer months of this section of North Carolina. Due to the type of spreading growth of the hydrogen-producing organisms (under the above climatic conditions) numerical counts of the colonies were further complicated since a surface colony might spread in a film over the surface of the agar, the same being true for the bottom colonies. However, in spite of the described cultural difficulties, it was found possible upon a number of occasions to identify colonies of these organisms from the routine, aerobic brine platings. This was facilitated after having become thoroughly familiar with the various forms of colonial growth.

During the 1938 season, the first attempt was made to reproduce the typical hydrogen fermentation by inoculation. A series of four 15 gal. kegs was divided into duplicate lots of two kegs each which were filled with discarded cucumber ends from the manufacture of fresh cucumber pickle. Both lots were salted at 60° salometer brine concentration and were maintained at that salinity. Inoculation was with 500 cc. of an actively fermenting, cucumber juice culture of one of the hydrogen-producing strains, growing at 20° salometer brine concentration. The brine pH of one lot was kept at 4.0 to 4.5, while the other was left unadjusted. Typical hydrogen fermentations resulted in the unadjusted lot while vigorous yeast fermentations developed in those in which the pH was controlled.

No claim is made with respect to complete reproduction of the hydrogen fermentations solely by inoculation since the organisms may have been present on the cucumbers in sufficient numbers to bring about such fermentation even though the ends received a thorough washing. This

possibility was clearly pointed out in the case of a 45 gal. cask of ends that was salted at the same time as the above lots. The brining was carried out at 50° salometer and a most vigorous hydrogen fermentation was found on the second day after having been put down. However, these inoculated fermentations are worthy of mention since they gave considerable opportunity for close observation of the fermentation as well as providing the source of some of the strains of the isolated organisms.

A total of 20 strains (Table 1, part A) were isolated by the anaerobic cultural methods from cucumber fermentations during the 1938 season. Of this number, eight were isolated from 40° salometer fermentations, nine from 50° salometer fermentations and three from 60° salometer fermentations. During the following season, nine additional strains were added to the collection of stock cultures (Table 1, part B). This group was divided as follows; two from 20° salometer fermentations and seven from 60° salometer fermentations. The strains isolated during 1938 were studied in detail, while those isolated during 1939 were used chiefly for comparative studies, particularly in reference to gas evolution and composition.

Table 1. Origin of Cultures Isolated from Cucumber Fermentations.

A. 1938 Season		B. 1939 Season	
Strain No.	Brine concentration	Strain No.	Brine concentration
	° sal.		° sal.
H-138	40	H-139	60
H-238	40		
H-338	40	H-239	20
H-438	40		
H-538	40	H-339	60
H-638	40		
H-738	40	H-439	60
H-838	40		
H-1138	60	H-539	60
H-1238	60		
H-1338	60	H-639	60
H-1438	50		
H-1538	50	H-739	20
H-1638	50		
H-1738	50	H-839	60
H-1838	50		
H-1938	50	H-939	60
H-2038	50		
H-2138	50		
H-2238	50		

IDENTIFICATION STUDIES

Twenty strains (Table 1, part A.) were studied in detail with respect to morphological, cultural and physiological characteristics. The procedure followed, for the most part, was according to the recommended bacteriological methods* (34). When a particular method for a test or procedure different from this was used, the reference is indicated by number.

During the study dealing with the cultural characteristics observed on the 10 solid and liquid media employed, complete observations with respect to the various characteristics were made for each of the 20 strains on each type of medium. This material was then analyzed and finally condensed into what might be called the typical characteristics for the type strain (H-1438) on the various media. This material is presented (Tables 3-11) in accordance with the prescribed methods (S.A.B.).

In the case of the biochemical or physiological tests, particularly where an observation is recorded as either positive or negative, no attempt was made to list such reactions, for all 20 strains since for the most part they were either of one reaction or the other. In cases where clear cut differences were noted, the number of strains reacting in each direction is pointed out. It will be noted that strains H-138 and H-238 showed the most variance from the typical characteristics representative of H-1438, particularly with reference to the following tests; methyl red, uric acid, and acid production.

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* Society of American Bacteriologists: Manual of Methods for Pure Culture Study of Bacteria. S.A.B., 7th ed., Geneva, N. Y., 1938.

The combined morphological, cultural and physiological characteristics of the 20 strains are presented in the summarized discussion of results.

For the sake of completeness, additional data are included in the summary of results (such as fermentation reactions on the various carbon compounds) although this information is not fully discussed until subsequent sections of this report.

After establishing the purity of the cultures by successive platings and prior to inoculation onto the cultural media, the cultures were given two transfers; first on nutritive caseinate agar (with 8.0 cc. of 0.4 per cent brom cresol purple per liter) and then in cucumber juice broth. From the latter, the inoculations onto the various media were made. A similar set of broth transfers was used at 24 hours of age for the morphological, staining and motility tests (Table 2).

Growth on the initial transfer stabs was best at the top, being gray, glistening and entire, with alkaline reaction extending down into the stab. Growth in the stab was out from the line of puncture and was lobate to papillate. An acid reaction prevailed in the deep part of the tube. Gas was formed in some cases in the base of the tube and in other cases was also present as small bubbles in the surface growth. When the gas formed in the base of the stab, the amount was sufficient to split the agar and raise the stab to the top of the tube.

Transfers made from these stabs into cucumber juice broth showed moderate growth in 12 hours and good to abundant growth in 24 hours. At this time a delicate pellicle or film appeared. The turbidity and the amount of sediment markedly increased during the 12 hour period. Gas

formation caused the broth to be heavily charged and when shaken, a foam of two to three cm. in height would rise up the tube. The broth remained turbid several days. If the pellicle was disturbed, so as to be submerged, a new one formed in 24 hours. The pellicle was glistening and sometimes flaky in appearance. Gas production was most vigorous during the first 24 to 48 hours.

At 72 hours of age the cucumber juice transfers, described above, were inoculated into the following media and incubated at 35° C. Observations were recorded at three days, one week and two weeks.

Culture medium	pH	Table reference No.
Nutritive caseinate agar slants (Difco)	6.6	3
Nutrient agar slants	6.8	4
Dextrose-tryptone agar slants (Difco)	6.8	5
Cucumber juice agar slants*	6.8	6
Cucumber juice broth*	6.6	7
Nutrient broth	6.8	8
Potato slants	...	9
Brom-cresol-purple milk	6.6	10
Plain gelatin stabs	6.8	11

* Expressed juice from fresh cucumbers adjusted to pH 6.8, no additional nutrients added. For solid medium, 15 g. of agar added per liter of juice.

Inasmuch as the descriptions (Tables 3-11) of the growth reactions on the various media are self explanatory they will not be discussed in detail but will be summarized later. However, attention is

called to a few of the more significant characteristics which are more or less typical of all strains. They are briefly as follows: (a) In nutritive caseinate agar stabs plus brom-cresol-purple, the surface growth appears to be about 0.5 cm. in area on the top of the stab; underneath, the reaction is alkaline about one-third the way down the tube and acid in the lower part of the tube and may be accompanied by gas production. (b) In plain agar slants, there is filiform growth with typical cross-hatching or net-like structure which is irridescent and translucent in character. Also, on this medium after about one week's time, there appears a spiked, feathery growth, down into the agar at irregular intervals along the slant. (c) In cucumber juice broth, there is rapid growth with pronounced turbidity and sediment. A delicate film or pellicle follows ring formation; the broth is heavily charged with gas, rising up in a foam when shaken. Cultures remain turbid for a number of days and have a large amount of sediment which is viscid in character. The odor of the cultures is of a sweetish character and tends to be aromatic, resembling a yeast fermentation. After prolonged incubation on nutrient agar, the odor tends to become mildly putrefactive. (d) Growth in brom cresol purple milk shows slight acidity at the first reading (3 days) which increased after the one week period and at two weeks there is coagulation with acid curd and evidence of trapped gas bubbles in the curd. (e) Gelatin stabs show slight to moderate growth at first, with a 0.5 cm. area of growth at the top of the stab. Lenticular pockets are present one-third the way down the tube. Later, at two weeks, liquefaction starts from the top and the surface of the gelatin drops down in a cone shape. Marked liquefaction is

found at three weeks. (g) Gas production in solid media containing dextrose is evidenced by the breaking up of the agar and the pushing up of the slants into the mouths of the tubes, also, by gas bubbles sometimes present directly in the surface growth on the slant.

The summarized data with respect to morphological, cultural and physiological characteristics are as follows:

Morphological

Rods, 0.5 x 1.0; 0.75 x 1.5; 1.0 x 2.5 microns, occurring singly, in pairs, in chains of three elements or masses and groups. Rods appear rounded on the ends and show indications of bi-polar staining. In all cases (20 strains) the majority of the cells are of the smaller size. In the masses and clumps, it appears that the cells are held together by a viscid material. They are gram negative, non-encapsulated and motile. In old cultures (eight months) the cells appear slightly smaller although no other changes were noted and no spores were found.

Cultural

Nutritive caseinate agar colonies: Growth on the surface may spread in a flat veily film covering the whole agar surface in the culture dish, also colonies may range from 1 to 7 cm. in diameter in fleecy, arborescent, branched filaments of growth which are irridescent and translucent. Sub-surface colonies are small, 0.5 x 1.0 x 2.0 mm. in size, lenticular in shape. However, growth on dextrose and plain agars (Figs. 2 and 3) may vary somewhat as to the colonial characteristics described above, chiefly with respect to spreading of the colonies and the branching type of growth. In general, growth on the latter media is more regular, without pronounced branched filaments.

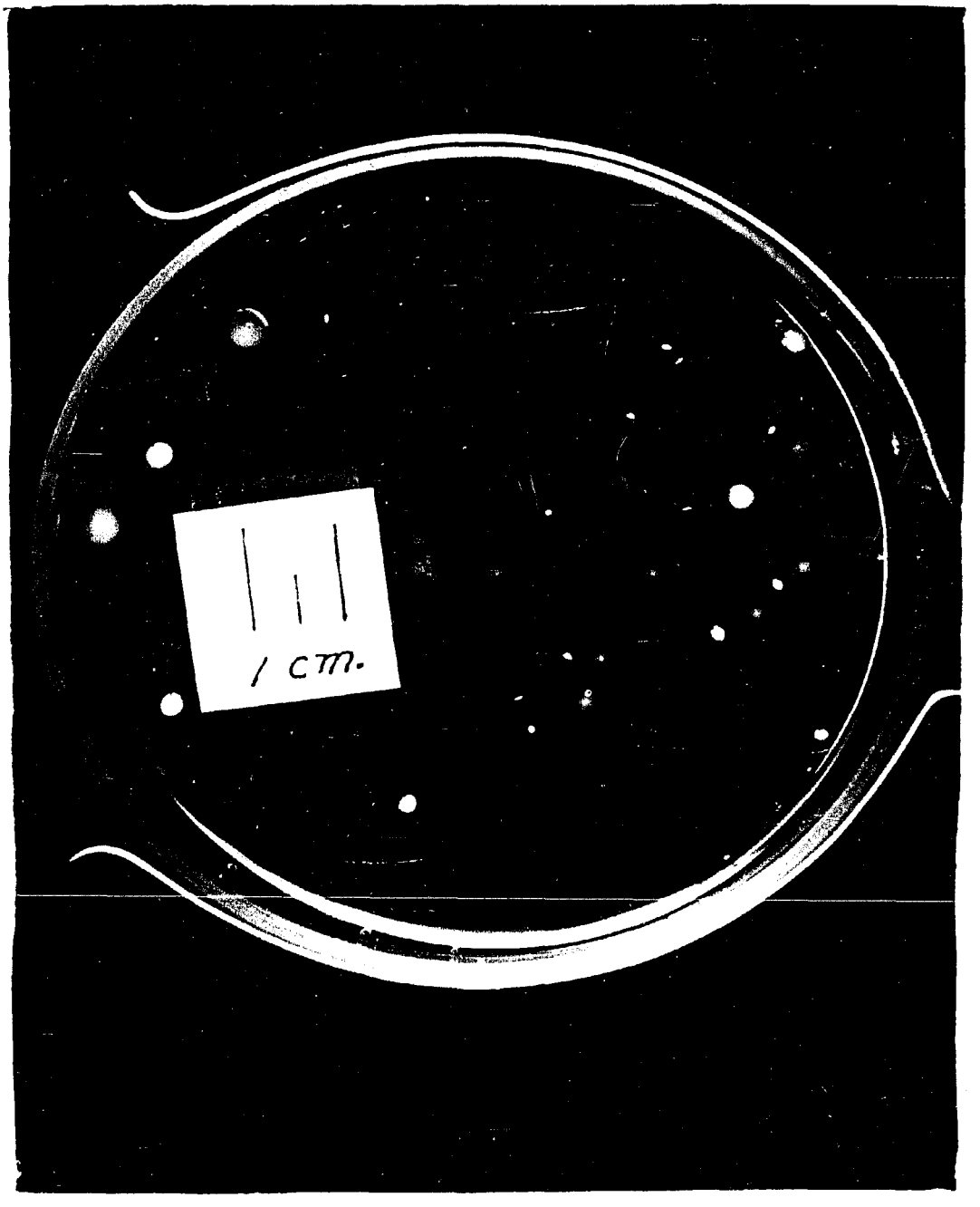

Fig. 2. Surface, sub-surface and bottom colonies from a stock culture of strain H-1438, 48 hours old, on nutrient agar.

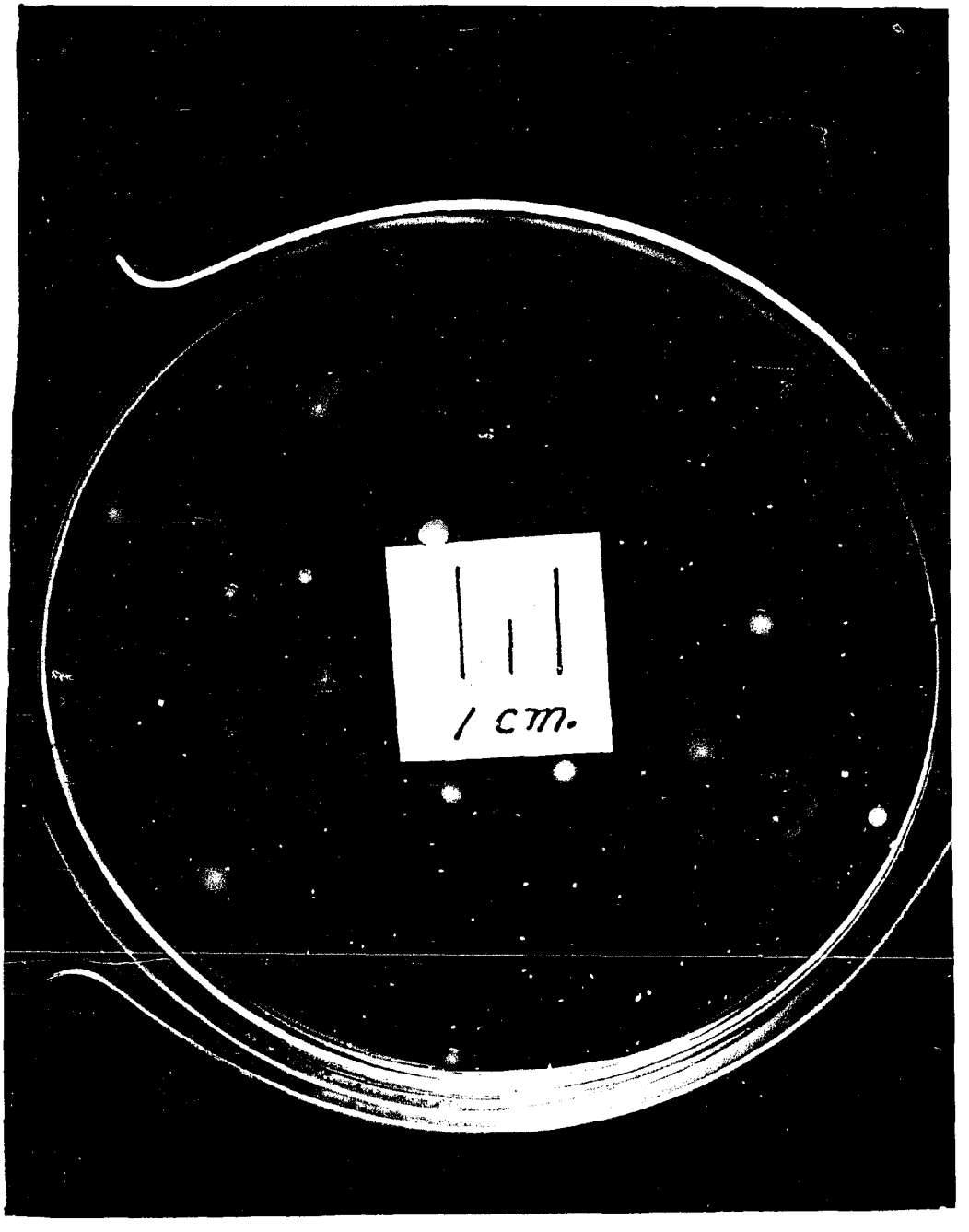

Fig. 3. Colonies of strain H-1438 after 48 hours on nutrient agar at 35° C. Note more spreading type of surface colonies, also bottom growth.

Nutritive caseinate agar slants: Growth abundant, filiform to slightly spreading at base of slant, edges finely lobate to wavy, glistening, flat, smooth to slightly contoured from center line, translucent, viscid to butyrous. Odor sweet to alcoholic. Acid reaction in butt of slant and alkaline beneath surface growth; a portion of the agar broken or split in bottom due to gas production.

Nutrient agar slants: Growth moderate to abundant (not as vigorous as that described for the above medium) filiform, spreading at base of slant, glistening, flat to somewhat contoured, finely reticulated with net-like cross-hatching (some strains smooth); irridescent, translucent with streak being white to grayish and being viscid to butyrous. No color to the medium, sweet to aromatic odor; no gas detected. After one week, cross-hatching or net-like appearance shows more clearly. Plates with numerous colonies, after six days incubation at room temperature have a mild putrefactive odor; also, the odor of ammonia can be detected. After two weeks, the majority of the strains show a spiked to feathery growth at irregular points along the streak, extending down into the agar.

Dextrose-tryptone agar slants: Growth abundant, filiform to spreading, smooth edges usually entire but may be wavy. Growth glistening, liquid in appearance, surface smooth and translucent as well as transparent. Streak grayish with viscid consistency, sweetish odor, acid in base of slant and alkaline underneath growth. Gas may be present in base of tube raising or breaking agar. After one week all strains show an alkaline reaction, in cases where the agar is broken away in the base of the tube, the lower portion shows a reduction of indicator to colorless.

Cucumber juice agar slants: Growth abundant, filiform with slight spreading in lower portion of slant. Some strains have a wavy, transparent periphery along the edge of the streak, glistening slightly convex, but generally smooth and flat; some strains wavy to moderately contoured, translucent, irridescent, gray to ivory color, viscid to butyrous and no apparent color given to medium; sweet to alcoholic odor. Gas production may split agar or push slant to top of tube, with bubbles being formed and trapped in surface growth. After one week some strains may develop the net-like, cross-hatched appearance described for the nutrient agar slants. After two weeks, growth appears more spreading in character, a secondary, arborescent growth showing from the edge of the primary streak.

Cucumber juice broth: Strong (3 plus) turbidity with delicate membrane or pellicle, which drops or disintegrates if disturbed. Moderate to abundant sediment, white, in masses, somewhat viscid and rises in a swirl. Gas present, the broth being heavily charged and if shaken a foam rises 3 to 4 cm. in height. After one week, pellicle (re-grown since tubes were shaken on the third day) has a glistening lustre. A perceptible sweetish odor observed. After two weeks a moderate clouding still exists, surface growth present but not continuous in all strains.

Nutrient broth: Slight to moderate turbidity (1 plus), membrane or delicate pellicle present in a few strains, also some with ring growth, the latter having villous projections down into the broth. The ring may fall in part into the broth. The majority of the strains are without surface growth. No odor detected. Small amount of sediment somewhat viscid in character when present in sufficient amounts to swirl.

No gas detected visually. After one week, slight to moderate persistent clouding. After two weeks, slight to moderate clouding, small amount of viscid sediment; no surface growth present.

Potato slants: Growth moderate to abundant, filiform, slightly irregular along the line of inoculation, glistening, smooth, slightly raised, yellowish to faint orange in varied degrees (for different strains), some appear ivory to cream in color; consistency slightly viscid to butyrous darkening the medium. Odor sweet to alcoholic. After one week, growth appears to be tan to golden. No change after two weeks.

Brom cresol purple milk (11): Slightly acid in reaction, acid curd absent, rennet curd absent, peptonization absent, film of growth at surface with gas bubbles trapped underneath. After one week an increased acid reaction, slight coagulation with one strain. After two weeks solid curd with all strains, some extrusion of whey pronounced acid reaction to indicator.

Plain gelatin stab: Growth filiform, finely papillate edges, gas bubbles in upper one-half to one-third of the stab along the line of inoculation; bubbles lenticular, 0.2 to 1.0 cm. in size. Liquefaction absent at four days. After one week the surface growth of 0.2 to 0.5 cm. area on top of the stab is smooth, grayish and glistening, gas bubbles still visible. After two weeks the gas bubbles disappear and the surface growth drops, leaving a sunken area. Liquefaction of napiform character, progressing more in some strains than in others although not pronounced being only 0.5 to 1.0 cm. in area and extending downward from 1.5 to 3.0 cm. After three weeks there is considerable liquefaction being complete in two strains, almost complete in 12 strains, and

about one-half complete in the other six strains being infundibuliform to stratiform in character.

Physiological

Indole (7): Not produced (by all strains).

Nitrates (41): Reduced (by all strains).

Hydrogen sulphide (43): Not produced (by all strains).

Catalase (17): Produced (all strains).

Methyl red test: Negative with majority of strains, a few doubtful, two (H-138 and 238) positive.

Voges-Proskauer test: Positive (all strains).

Uric acid test (2): Uric acid can be utilized as the sole source of nitrogen by most strains. H-138 and 238 doubtful.

Citric acid test (2): Citric acid can be utilized as the sole source of carbon by all strains.

Effect of organic acids: Growth usually inhibited by about 0.05 per cent acetic acid and 0.1 per cent lactic acid.

Cleavage of carbon compounds: Demonstration of cleavage by evolution of gas from: l-arabinose, dextrose, d-galactose, lactose, levulose, maltose, d-mannose, d-mannitol, raffinose, rhamnose, saccharose, salacin, d-sorbitol, and l-xylose; cleavage was demonstrated for the following additional compounds by the indicated number of strains: dextrin, 3; glycerol, 14; inulin, 3; starch, 11; lemon pectin, 2. Melezitose was not attacked by any of the strains. Likewise, cellulose was not attacked by the strains tested. The detailed observations for the above compounds, with the exception of pectin and cellulose, are shown in Table 12.

Relation to oxygen: Aerobic, facultative, will grow abundantly under both aerobic and anaerobic conditions. Anaerobic growth demonstrated by cultivation in solid and liquid media in an anaerobic jar (large desiccator) as described in Fig. 1.

Relation to salt: Growth in concentrations upwards of 80° salometer (21.1 per cent salt) in normal cucumber fermentations. However, under ordinary laboratory conditions, using liquid media, growth is not observed visually in salt concentrations exceeding about one-half the above amount. Some strains, may be made to increase in salt tolerance when sub-cultured serially in liquid media containing salt. Salt is not necessary in ordinary media for abundant growth.

Thermal death time: Instantaneous to six minutes exposure at 60° C., depending on the strain. Cultures able to withstand 50° C. for 10 minutes.

Optimum growth temperature: 30 to 35° C. No gas produced at 45° C. although growth may be noted.

Optimum pH conditions: Maximum gas production in a buffered series, 5.1 to 5.3. Excellent gas production occurred in unbuffered dextrose broth, equal to or exceeding that of the buffered lot.

The foregoing data with respect to morphological, cultural and physiological characteristics (as well as the biochemical studies on acid production and end-products of the fermentation which are discussed shortly) assure positive identification of the 20 cultures as belonging in the genus Aerobacter (Beijerinck) described in Bergey's manual (6). Only two true species of the genus are recognized in the above manual, they are, Aerobacter aerogenes and Aerobacter cloacae. Both species have

a great number of characteristics in common; however, they differ chiefly with regard to fermentation of glycerol and liquefaction of gelatin. With A. aerogenes glycerol is fermented and gelatin is not liquefied while with A. cloacae the reverse is true for the action on both compounds.

Taking into account the above reactions as well as other characteristics of the group as a whole, 18 of the 20 cultures can be considered allied to the species cloacae although the characteristics are by no means identical. Within the group are a number of cultures that by virtue of their action on glycerol and starch as well as other characteristics may be considered as intermediates and allied to both the species of the genus Aerobacter, namely, A. aerogenes and A. cloacae. The remaining two cultures (H-138 and H-238) must be considered as further varieties of A. cloacae.

Acid production

When viewed in the light of known behavior of the Aerobacter, the results of the following investigation upon acid production from various compounds by all strains becomes more understandable.

The test solutions listed in Table 12 were titrated at the conclusion of the incubation period (two weeks) and the values with respect to amounts of N/10 acid or alkali required for neutralization are presented in Table 13. In the majority of the cases, the final reaction to the indicator was alkaline, necessitating titration with acid. Only two strains, H-138 and H-238 (varients) showed any consistency in fermenting the compounds with resulting acid production which was still present at the time of analyses. In general, it would appear that for

the majority of the strains tested, the final acidity after two weeks incubation was negligible or absent. The results of a subsequent investigation, dealing with the acid production from dextrose after four days incubation (Table 14), serve to confirm the preceding data. In the case of the fermentation of dextrose, out of the 29 cultures studied, 14 or 48 per cent showed negative values after correcting for the control. The balance of the cultures (with the exception of H-138 and H-238) were either neutral or nearly so.

If appreciable amounts of acid were produced by the Aerobacter cultures, other than the exceptions noted, it was either neutralized by alkaline compounds formed or it was destroyed to form other compounds. Previous work points toward the latter condition being brought about. The reversion of acidity caused by the Aerobacter has been shown by Ayers and Rupp (5). Also, these workers pointed out that this reversion could be accomplished under conditions which were independent of alkali production. Furthermore, they demonstrated that the acetic acid formed by the organisms during the dextrose fermentation was in part destroyed. The findings of Reynolds and Werkman (30) confirmed those of Ayers and Rupp and in addition showed that the reversion of the accumulated acetic acid was accompanied by an increase in acetylmethylcarbinol and 2, 3-butylene glycol brought about by condensation and reduction.

End-products of the fermentation

It has been shown (18, 32) that the fermentation of dextrose by Aerobacter aerogenes yielded formic, lactic and succinic acids, as well as carbon dioxide, hydrogen, ethyl alcohol, 2, 3-butylene glycol, and acetylmethylcarbinol. Of these products, it has been suggested (32)

that succinic acid probably resulted from the protein present in the culture medium. In an investigation of the dissimilation products of dextrose by Aerobacter indologenes (regarded by the authors of Bergey's manual as a variety of A. cloacae), Reynolds and Werkman (30) showed that the following products were formed; formic, acetic and lactic acids, ethyl alcohol, hydrogen, carbon dioxide, acetylmethylcarbinol and 2, 3-butylene glycol. Of these products, formic and acetic acids and acetylmethylcarbinol decreased in amount subsequent to prior accumulation. The formic acid was decomposed to carbon dioxide and hydrogen; the acetic acid to acetylmethylcarbinol and then the latter to 2, 3-butylene glycol.

It is evident from the above discussion that the products from the fermentation of dextrose by both species of the genus Aerobacter are essentially the same.

In the present studies, an investigation of a limited nature upon the fermentation of dextrose by type strain H-1438 (see Table 15) showed the following products were formed; carbon dioxide, hydrogen, ethyl alcohol and esters (trace). Also, the fermented liquor gave a strong test (qualitative) for the presence of acetylmethylcarbinol. The determination for butyl alcohol was negative. The fermentation by strain H-138 (variant), resulted in the production of non-volatile acids (probably, chiefly lactic) and volatile acids in addition to the products mentioned for strain H-1438.

In view of the earlier discussion of the destruction of acetic and formic acids by the Aerobacter it is quite probable that this behavior accounts for the absence of acids in the case of the four day fer-

mentation by strain H-1438. If such is to be presumed, then it is not at all unlikely that little or no lactic acid was produced, since reversion of the acidity was complete. However, with the fermentation by the variant (H-138) the above relationship with regard to reversion of acidity was not pronounced since an appreciable amount of non-volatile acid as well as a determinable amount of volatile acid was found at the conclusion of the four-day fermentation period.

Table 2. Morphological Characteristics of the Cultures, Including Motility Determinations.

Culture No.	Gram Stain	Motility (broth)	Size ranges in microns and arrangement
H-138	-	+	Rods: .75 x 1; 1 x 2; 1 x 3; occurring singly, some pairs, 3's and clumps and masses.
H-238	-	+	Rods: .75 x 1; 1 x 3; 1 x 4; occurring mostly singly, few pairs, also in groups and clumps.
H-338	-	+	Rods to cocco-bacilli: .75 x .75 to 1; few 1 x 3; almost oval, occurring singly, pairs and clumps.
H-438	-	+	Rods: .75 x 1; 1 x 1.25; 1 x 2.5; occurring singly, pairs and clumps. (evidence of bipolar staining.)
H-538	-	+	Rods: .75 x 1; 1 x 2.5; mostly singly, also in clumps and masses, some in 2's and 3's and groups.
H-638	-	+	Same as H-538
H-738	-	+	Rods: .75 x 1; .75 x 2; .75 x 2.5; occurring singly, 2's, 3's and in groups and masses and clumps.
H-838	-	+	Rods: .75 x 1.25; 1 x 2.0; singly, 2's, groups, masses and clumps.
H-1138	-	+	Rods: .50 x 1; .75 x 1.5; 1 x 2.0; occurring singly, 2's, groups, masses and clumps; some cells cocci-to oval.
H-1238	-	+	Rods: .75 x 1; 1 x 1.5; .75 x 2; 1 x 3; occurring singly, 2's, clumps and masses. Most cells the smaller size.
H-1338	-	+	Rods: .50 x 1; .75 x 1.5; .75 x 2.5-3; smaller size in the majority; occurring singly, clumps, groups and masses.
H-1438	-	+	Rods: .50 x .75; 1 x 1.25; 1 x 3; occurring singly, 2's, clumps, masses and groups.
H-1538	-	+	Rods: .50 x .75; .75 x 1.5; 1 x 2; few .75 x 4; occurring singly, pairs, clumps and masses.

Table 2. Morphological Characteristics of the Cultures, Including Motility Determinations (continued).

Culture No.	Gram Stain	Motility (broth)	Size ranges in microns and arrangement
H-1638	-	+	Rods: .50 x .75; .75 x 1.5; .75 x 3; occurring singly, pairs, clumps, groups and masses.
H-1738	-	+	Rods: .50 x .75; .75 x 1.25; .75 x 2; singly, pairs, groups and masses.
H-1838	-	+	Rods: .50 x .75; small-to oval appearance the majority, also some .75 x 2 to 3; occurring singly, pairs and masses.
H-1938	-	+	Rods: .50 x .75; .75 x 2; .75 x 3; occurring singly, pairs, 3's, clumps and masses.
H-2038	-	+	Rods: .50 x .75; .75 x 1.5; .75 x 2-3; occurring singly, pairs, masses, groups and clumps.
H-2138	-	+	Rods: .75 x 1-1.5; 1 x 2.5; occurring singly, pairs, short chains, groups and masses.
H-2238	-	+	Rods: .75 x 1-1.5; 1 x 2; few 1 x 3; singly, pairs, groups, masses, and clumps. Most cells smaller size.

N. B. The rods appear rounded on the ends and show indications of bipolar staining. In all cases the majority of the cells are of the smaller sizes listed. In the masses or clumps it appears that the cells are held together by a viscid material. Spores absent.

Table 3. Growth of Cultures, Nos. H-138 Through 2238 on NUTRITIVE CASEINATE AGAR SLANTS After three days Incubation at 35° C.

GROWTH:	Abundant to moderate
FORM:	Filiform to slightly spreading at the base of growth. Edges finely lobate to wavy.
LUSTRE:	Glistening
ELEVATION:	Flat
TOPOGRAPHY:	Smooth to slightly contoured from center line.
OPTICAL CHARACTERISTICS:	Translucent; irridescence not noted on this medium.
CHROMOGENESIS:	Grayish
CONSISTENCY:	Viscid to butyrous
COLOR TO MEDIUM:	Not determined, B.C.P. in the agar.
ODOR:	Sweet to alcoholic
REACTION:	Acid in the butt and alkaline beneath surface growth.

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AFTER ONE WEEK INCUBATION

Growth filiform, starting to spread from the base upward, growth being smooth to contoured along the edges, the latter being finely lobate and appearing wavy. Alkaline reaction in all tubes except H-2238, 1538, 338 and 138; of these, all but 338 have acid reaction in the portion of the slant broken away by gas production. These are the strains showing evidence of gas.

AFTER TWO WEEKS INCUBATION

Same as for the one week observation with all tubes having alkaline reaction. Cultures have sweetish odor, resembling esters.

Table 4. Growth of Cultures, Nos. H-138 Through 2238 on NUTRIENT AGAR SLANTS After three days Incubation at 35° C.

GROWTH:	Moderate to abundant, not as good as on cucumber juice
FORM:	Filiform, some strains spreading in base of slant
LUSTRE:	Glistening
ELEVATION:	Flat
TOPOGRAPHY:	Contoured, finely stippled to net-like cross-hatching, some strains smooth.
OPTICAL CHARACTERISTICS:	Irridescent, translucent
CHROMOGENESIS:	Streak being white to grayish
CONSISTENCY:	Viscid to butyrous
COLOR TO MEDIUM:	None
ODOR:	Sweetish odor
GAS:	None detected visually

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AFTER ONE WEEK INCUBATION

Same as for the three day description, a little more growth present. Most strains spreading at the base, clearly showing cross-lines or net-like appearance. Odor mildly putrefactive in character.

AFTER TWO WEEKS INCUBATION

Similar to one week observation. Noted that the majority of the cultures showed a spiked to feathery growth at irregular points along the growth that extend down into the agar. Note:- The same spiked, feathery growth noted for cucumber juice agar but longer and more filamentous in nature. This is not present in the nutritive caseinate agar slants nor in the dextrose tryptone agar slants.

Table 5. Growth of Cultures, Nos. H-138 Through 2238 on DEXTROSE TRYPTONE AGAR SLANTS After three days Incubation at 35° C.

GROWTH:	Abundant
FORM:	Filiform to spreading, smooth edges, usually entire, may be wavy.
LUSTRE:	Glistening, liquid appearance
ELEVATION:	Flat
TOPOGRAPHY:	Smooth
OPTICAL CHARACTERISTICS:	Translucent and transparent
CHROMOGENESIS:	Grayish
CONSISTENCY:	Viscid
COLOR TO MEDIUM:	Not determined, B.C.P. in medium
ODOR:	Sweetish, resembling esters
REACTION:	Acid in base or butt, alkaline beneath a stroke.
GAS:	May be present in base of tube, raising the agar or may break agar or crack it.

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AFTER ONE WEEK INCUBATION

All tubes show alkaline reaction, somewhere the agar is broken away at the bottom and having gas present show a reduction of the indicator (B.C.P.) to colorless. Growth is spread across the slant up to the top one-third of the tube and growth appears to be glistening and liquid, growth is of viscid consistency.

AFTER TWO WEEKS INCUBATION

Same as for the one week observations.

Table 6. Growth of Cultures, Nos. H-138 Through 2238 on CUCUMBER JUICE AGAR SLANTS After three days Incubation at 35° C.

GROWTH:	Most abundant
FORM:	Filiform with slight spreading in lower part of streak; some strains have a wavy, transparent periphery along the streak.
LUSTRE:	Glistening
ELEVATION:	Slightly convex
TOPOGRAPHY:	Smooth generally, some wavy, moderately contoured.
OPTICAL CHARACTERISTICS:	Translucent, irridescent
CHROMOGENESIS:	Gray to ivory in color
CONSISTENCY:	Viscid to butyrous
COLOR TO MEDIUM:	None detected
ODOR:	Sweet to alcoholic
GAS:	May split the agar or push the slant to the top of the tube, gas bubbles may occur in the growth.

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AFTER ONE WEEK INCUBATION

Description for the third day similar to the one week results. Streaks may be filiform or spreading. Filiform growth slightly convex. Some strains (338, 438, 1838, and 1938) have a wrinkled, cross-hatched, net-like growth described originally for the nutrient agar slants. All strains are glistening and irridescent. Odor and consistency the same as third day.

AFTER TWO WEEKS INCUBATION

Same as for the one week, may be a tendency for more spreading growth which is complete in some, growth from the edge of the growth being aborescent in character.

Table 7. Growth of the Cultures, Nos. H-138 Through 2238 in CUCUMBER JUICE BROTH After three days Incubation at 35° C.

SURFACE GROWTH:	Film to membrane or pellicle, delicate in nature drops or disintegrates if disturbed.
CLOUDING:	Strong, considerable turbidity. (+++)
ODOR:	Resembling cucumber juice broth
SEDIMENT:	White, masses, somewhat viscid and rises in a swirl of masses.
AMOUNT OF SEDIMENT:	Moderate to abundant.
TURBIDITY:	3+
GAS:	Present
REMARKS:	The broth of the type strain is heavily charged with gas; if shaken, foam rises 3 to 4 cm. up the tube.

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AFTER ONE WEEK INCUBATION

Film or pellicle present and of glistening lustre, this growth regrown since the tubes were shaken on the third day when the tubes were shaken to determine the character of the sediment. Strains H-238 and 438 beginning to clear. Moderate to abundant sediment in most tubes. Slight sweetish odor observed. Balance of description same.

AFTER TWO WEEKS INCUBATION

Similar to the one week incubation description, still moderate clouding and surface growth but not necessarily continuous.

Table 8. Growth of Cultures, Nos. H-138 Through 2238 in NUTRIENT BROTH After three days Incubation at 35° C.

SURFACE GROWTH:	Membrane or delicate pellicle in a few strains, also a few strains with ring growth having villous projections down into the broth, the ring may fall in part down into the broth. Majority of the culture with no surface growth.
CLOUDING:	Slight to moderate
ODOR:	None detected
SEDIMENT:	Small amount, somewhat viscid in character when enough to swirl.
AMOUNT OF SEDIMENT:	Small
TURBIDITY:	1+
GAS:	None detected visually.

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AFTER ONE WEEK INCUBATION

Description similar to those of the third day. Type strains have persistent clouding, slight to moderate; H-138, 238 and 338 clouding is slight, the named cultures being the only ones having any trace of surface growth in the form of ring. The balance of the cultures have no surface growth. Sediment viscid, small amount.

AFTER TWO WEEKS INCUBATION

Same as for the one week observation, slight to moderate clouding, being persistent. Small amount of sediment which is viscid. No surface growth.

Table 9. Growth of Cultures, Nos. H-138 Through 2238 on POTATO SLANTS After three days Incubation at 35° C.

GROWTH	Moderate to abundant
FORM:	Filiform, slightly irregular along line of inoculation
LUSTRE:	Glistening
TOPOGRAPHY:	Smooth
ELEVATION:	Slightly raised
CHROMOGENESIS:	Yellowish to faint orange in varied degrees in different strains. Some appear ivory to cream color.
CONSISTENCY:	Slightly viscid to butyrous
COLOR TO MEDIUM:	Darkened, yellowish to brownish
ODOR:	Sweet with alcoholic aroma

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AFTER ONE WEEK INCUBATION

Similar to three day description, no more growth noticeable. Characteristics similar. Color seems to be tan to light golden color.

AFTER TWO WEEKS INCUBATION

Same as for the one week description.

Table 10. Growth of Cultures, Nos. H-138 Through 2238 in BROM-CRESOL PURPLE MILK After three days Incubation at 35° C.

REACTION:	3 days, slightly acid; 14 days, acid.
ACID CURD:	3 days, 0; 14 days +.
RENNET CURD:	3 days, 0; 14 days 0.
PEPTONIZATION:	3 days, 0; 14 days 0.
REMARKS:	Indicator changed to acid reaction, that of a yellowish color. There was a film or pellicle at the surface with gas bubbles trapped underneath.

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AFTER ONE WEEK INCUBATION

Increased acid reaction over the three days observation, + to +++. Slight coagulation in the lower part of H-1838. Other strains the same as at the three days observation except for more acid production. No changes in consistency.

AFTER TWO WEEKS INCUBATION

Solid curd in all tubes with or without extrusion of whey or presence of gas bubbles. Acid reaction to the indicator.

AFTER THREE WEEKS INCUBATION

All strains have formed acid curd and gas bubbles or pockets visible in the curd, some extrusion of whey.

Table 11. Growth of Cultures, Nos. 138 Through 2238 in PLAIN GELATIN STABS After four days Incubation at 35° C.

GROWTH:	Uniform, 0.25 to 0.50 diameter area on surface of stab.
LINE OF PUNCTURE:	Filiform, finely papillate edges. Gas bubbles in the upper one-half to one-third part of the stab along the line of inoculation, bubbles lenticular, 0.2 to 1.0 cm. in diameter.
LIQUEFACTION:	Absent at four days.
DEGREE:	None
METHOD USED:	Stabs kept at 20° C. After inoculation they were kept at the above temperature in water bath.
COLOR TO MEDIUM:	Unchanged.

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AFTER ONE WEEK INCUBATION

Growth uniform, 0.2 to 0.5 cm. area on top of slant, smooth grayish, glistening. Filiform in character with finely papillate edges which are wavy out from the stab growth. Gas bubbles present at the three day observation still visible; no sign of liquefaction at one week.

AFTER TWO WEEKS INCUBATION

At two weeks the gas pocket disappeared and the top growth on the gelatin dropped leaving a sunken area. Liquefaction in napiform character and more progressed in some strains than in others. Gas pocket areas becoming liquefied from the sunken top of gelatin.

Table 12. Gas Production from Various Carbon Compounds (Carbohydrates, Alcohols and Glucosides).

Methods: Compounds (1.0 per cent) made up in basal broth of 0.5 per cent tryptone and 0.2 per cent yeast extract dispensed in 5 cc. amounts into tubes containing insert tubes (4.5 x 0.8 cm.) and sterilized (10 lbs. pressure for 10 min.)

Figures shown below are expressed as per cent volume of the insert tubes and represent one-tenth of actual production. Each set of four figures (left to right) indicate production of gas at 48 hours, 84 hours, one week and two weeks.

	CARBON COMPOUNDS				
Culture No.	Arabinose	Dextrose	Dextrin	Glycerol	Galactose
H-138	4-2-2-2-	4-2-2-1	1-1-1-+	0-0-0-0	5-4-4-3
H-238	3-2-2-2	3-2-2-2	0-0-0-0	0-0-0-0	4-3-3-3
H-338	8-6-4-3-	6-4-3-2-	0-0-0-0	0-+-1-1	7-6-5-4-
H-438	9-9-3-2	9-7-3-2	0-0-0-0	0-0-0-0	7-10-6-4
H-538	7-4-4-2	7-6-2-1	0-0-0-0	0-0-0-0	7-9-9-6
H-638	10-7-3-2	8-5-2-1	0-0-0-0	0-+-+-+	6-6-3-3
H-738	7-5-2-1	10-7-3-2	0-0-0-0	0-±-+-+	5-9-8-4
H-838	10-4-4-2	6-7-2-1	0-0-0-0	0-0-0-0	10-10-7-5
H-1138	10-4-4-3	6-7-1-1	0-0-0-0	0-±-+-+	6-5-3-2
H-1238	6-6-3-1	10-7-4-2	0-0-0-0	0-0-0-0	6-8-9-6
H-1338	7-4-2-1	7-4-2-2	0-0-0-+	0-0-+-+	8-10-6-4
H-1438	7-3-2-1	7-4-2-1	0-0-0-0	±-1-1-1	10-8-5-3
H-1538	7-4-3-+	7-5-3-+	0-0-0-0-	1-1-1-1	5-4-2-1
H-1638	6-3-2-+	8-5-3-1	0-0-0-0	±-1-1-1	10-10-7-3
H-1738	8-4-2-1	9-6-3-2	0-0-0-0	0-±-+-+	10-7-4-3
H-1838	10-7-4-2	5-3-1-1-	0-0-0-0	0-+-+-+	7-5-3-1
H-1938	5-6-5-2	10-7-4-3	0-0-0-+	0-±-+-+	8-10-10-6
H-2038	4-3-1-1	7-4-2-1	0-0-0-0	0-+-1-+	10-10-7-3
H-2138	7-4-2-2	6-3-2-1	0-0-+-+	±-1-1-1	7-3-2-1
H-2238	7-4-3-1	4-2-1-1	0-0-0-0	0-+-+-+	8-6-3-2
Uninoc. Controls	0	0	0	0	0

See footnotes at end of table.

Table 12. Gas Production from Various Carbon Compounds (Carbohydrates, Alcohols and Glucosides) (Continued).

Culture No.	Inulin	Lactose	Levulose	Maltose	Mannose
H-138	+-+-+-0	1-3-5-7	3-3-2-1	3-2-2-1	8-6-6-5
H-238	0-0-0-0	3-4-3-4	7-6-4-3	3-2-2-3	6-5-4-3
H-338	0-0-0-0	4-10-10-7	4-3-1-1	8-9-10-8	8-6-4-3
H-438	0-0-0-0	7-8-9-6	10-6-3-2	10-10-7-5	8-5-2-2
H-538	0-0-0-0	1-3-5-4	10-9-6-3	5-8-9-7	8-5-3-1
H-638	0-0-0-0	3-7-8-6	10-8-5-3	10-10-7-6-	6-4-2-1
H-738	0-0-0-0	0-1-2-3	10-7-4-2	10-10-10-7	10-10-7-3
H-838	0-0-0-0	2-5-7-6	10-6-3-2	6-8-10-7	10-8-4-2
H-1138	0-0-0-0	2-3-3-5	10-8-4-3	9-10-10-6	7-4-3-1
H-1238	1-1-+-+	0-3-5-6	10-7-3-2	10-10-9-6	10-7-3-2
H-1338	0-0-0-0	3-8-9-6	4-2-1-1	7-8-7-4	9-6-3-2
H-1438	0-0-0-0	5-8-10-5	10-7-4-2	10-10-9-4	10-8-6-3
H-1538	0-0-0-0	5-7-9-6	7-4-2-1	10-10-9-6	8-6-4-2
H-1638	0-0-0-0	4-5-6-5	10-6-3-2	10-10-10-5	8-5-3-1
H-1738	0-0-0-0	9-10-6-4	9-5-3-2	10-8-6-4	10-6-2-1
H-1838	0-0-0-0	5-8-10-6	7-4-2-1	7-7-5-3	7-4-1-1
H-1938	0-0-0-0	1-2-3-3	6-3-2-1	6-7-7-4	8-5-3-2
H-2038	0-0-0-0	5-7-9-9	10-10-5-2	10-10-6-4	10-8-5-2
H-2138	1-1-+-1	5-7-8-5	10-6-4-2	10-10-9-5	8-7-4-2
H-2238	+-0-0-0	3-6-6-3	7-4-2-1	7-7-6-3	7-4-2-1
Uninoc. Controls	0	0	0	0	0

See footnotes at end of table.

Table 12. Gas Production from Various Carbon Compounds (Carbohydrates, Alcohols and Glucosides) (Continued).

Culture No.	Mannitol	Melezitose	Raffinose	Rhamnose	Saccharose
H-138	7-5-4-3	0-0-0-0	4-3-1-3	2-2-2-1	4-4-3-2
H-238	7-6-5-3	0-0-0-0	4-3-3-5	1-1-1-+	2-2-2-2
H-338	7-7-4-3	0-0-0-0	3-3-3-3	2-2-2-1	8-7-3-2
H-438	8-5-2-2	0-0-0-0	8-8-7-5	2-3-1-1	10-8-5-3
H-538	7-7-7-4	0-0-0-0	7-8-9-7	3-3-2-1	5-3-2-1
H-638	6-7-4-3	0-0-0-0	10-10-7-6	3-3-2-1	7-4-2-1
H-738	10-7-6-3	0-0-0-0	6-7-10-5	2-2-1-+	8-7-6-4
H-838	10-7-5-4	0-0-0-0	3-3-5-3	2-2-1-1	7-4-2-1
H-1138	7-6-3-2	0-0-0-0	8-10-10-6	2-3-2-1	10-7-3-2
H-1238	10-10-6-3	0-0-0-0	6-7-4-5	2-2-7-1	7-4-1-1
H-1338	6-5-3-2	0-0-0-0	5-6-6-4	1-1-+-+	7-6-4-2
H-1438	10-8-5-3	0-0-0-0	7-9-10-7	2-3-3-2	7-5-3-2
H-1538	9-7-5-5	0-0-0-0	3-4-4-3	2-3-4-3	8-10-8-6
H-1638	10-8-5-4	0-0-0-0	6-6-6-4	+-+-+-+-+	8-8-7-3
H-1738	10-6-3-2	0-0-0-0	6-6-7-4	1-1-+-+	10-7-7-3
H-1838	10-7-5-4	0-0-0-0	6-6-8-4	±-+-+-+	8-7-4-2
H-1938	7-9-7-4	0-0-0-0	2-3-3-4	1-+-+-+	4-3-1-1
H-2038	10-8-5-3	0-0-0-0	6-7-7-7	1-1-1-+	8-8-6-3
H-2138	10-7-6-4	0-0-0-0	4-4-5-5	2-2-3-2	8-7-6-4
H-2238	9-7-4-3	0-0-0-0	4-5-7-5	1-1-+-+	8-6-4-2
Uninoc. Controls	0	0	0	0	0

See footnotes at end of table.

Table 12. Gas Production from Various Carbon Compounds (Carbohydrates, Alcohols and Glucosides) (Continued).

Culture No.	Salacin	Sorbitol	Starch	Xylose	Broth Control
H-138	3-4-3-3	5-7-7-7	1-1-1-1	5-5-4-3	0-0-0-0
H-238	3-4-3-3	4-7-7-6	1-1-1-2	4-4-3-3	0-0-0-0
H-338	1-1-2-2	7-8-10-8	1-1-2-2	7-7-7-7	0-0-0-0
H-438	7-6-2-2	9-10-5-4	0-0-0-0	7-7-7-7	0-0-0-0
H-538	6-5-4-3	10-10-8-7	0-0-0-0	7-7-7-8	0-0-0-0
H-638	5-4-2-2	8-9-7-6	0-0-0-0	7-8-7-7	0-0-0-0
H-738	8-7-6-4	8-10-9-7	0-0-0-0	6-7-7-7	0-0-0-0
H-838	7-6-4-3	8-7-7-5	0-0-0-0	6-8-8-8	0-0-0-0
H-1138	7-5-3-2	7-10-10-6	0-0-0-0	7-8-8-6	0-0-0-0
H-1238	7-7-7-4	8-10-8-6	1-+-+-+	8-8-8-8	0-0-0-0
H-1338	6-5-4-3	8-10-8-6	lost	8-8-9-9	0-0-0-0
H-1438	2-3-3-3	10-10-8-4	1-1-1-1	8-10-10-10	0-0-0-0
H-1538	2-3-3-3	10-8-7-6	0-0-0-1	8-9-10-10	0-0-0-0
H-1638	7-7-5-3	9-9-8-6	0-0-0-+	8-8-8-6	0-0-0-0
H-1738	6-6-3-3	7-10-5-6	0-0-0-+	7-7-8-8	0-0-0-0
H-1838	7-5-3-2	9-8-7-4	1-1-1-1	6-7-7-8	0-0-0-0
H-1938	1-2-2-2	7-9-10-8	1-1-1-1	7-6-6-6	0-0-0-0
H-2038	6-4-3-2	9-10-8-6	0-±-0-0	8-8-8-8	- - -
H-2138	2-3-3-4	10-10-8-7	1-1-1-1	7-8-9-8	0-0-0-0
H-2238	2-3-3-3	9-10-7-6	0-0-0-0	8-8-8-6	0-0-0-0
Uninoc. Controls	0	0	0	0	0

\+ Indicating gas present, not sufficient to measure.
0 No gas evident.
1 10 per cent gas; 2, 20 per cent gas, etc.
± Indicating doubtful gas production, very small bubble.
Note: In most cases, the fermentation is rapid, the maximum volume of gas is produced within about 48 hrs. After this period the volume decreases due to the great solubility and rapid diffusion of carbon dioxide. The remaining portion of gas may be assumed to be hydrogen since later studies showed only two gases produced.

Table 13. Acid Production from Various Carbon Compounds (Carbohydrates, Alcohols and Glucosides) After two weeks Incubation at 35° C.

Methods: Same medium as outlined in Table 12 including preparation of carbon compounds. At conclusion of two weeks the contents (5 cc.) of each tube were titrated with N/10 NaOH, using phenolphthalein as the indicator. Where the cultures gave an alkaline reaction to the indicator an estimation of the amount of alkali was determined by use of N/10 HCl.

	CARBON COMPOUNDS						
Culture No.	Arab-inose	Dex-trose	Dex-trin	Glyc-erol	Galac-tose	Inu-lin	Lac-tose
H-138	0.8*	0.6*	0.5	0.7	1.0*	1.3	0.0
H-238	0.4	0.8*	0.5	0.1	0.9*	0.5	0.1*
H-338	0.7	0.7	0.5	0.2*	0.6*	0.5	0.5
H-438	0.6	0.7	0.5	0.3*	0.6	0.5	0.6
H-538	0.7	0.7	0.5	0.1*	0.6	0.5	0.6
H-638	0.7	0.7	0.4	0.3	0.7	0.5	0.7
H-738	0.7	0.7	0.2	0.1	0.6	0.5	0.2
H-838	0.8	0.8	0.5	0.2*	0.7	0.5	0.5
H-1138	0.7	0.7	0.6	0.1	0.7	0.5	0.2
H-1238	0.7	0.8	0.2	0.0	0.5	0.6	0.2
H-1338	0.7	0.8	0.4	0.2	0.7	0.6	0.8
H-1438	0.8	0.8	0.5	0.1*	0.3	0.3*	0.7
H-1538	0.7	0.7	0.2	0.3*	0.8	0.3*	0.5
H-1638	0.7	0.7	0.2	0.1	0.8	0.4*	0.2
H-1738	0.7	0.7	0.2	0.1	0.7	0.5	0.8
H-1838	0.7	0.7	0.4	0.1	0.7	0.4*	0.6
H-1938	0.6	0.8	0.6	0.1*	0.6	0.5	0.2
H-2038	0.8	0.7	0.5	0.1	0.7	0.3*	0.1
H-2138	0.7	0.7	0.6	0.0	0.7	0.3*	0.6
H-2238	0.6	0.7	0.1	0.0	0.7	0.6	0.6
Uninoc. Controls	0.4*	0.4*	0.4*	0.3*	0.4*	0.3*	0.4*

See footnote at end of table.

Table 13. Acid Production from Various Carbon Compounds (Carbohydrates, Alcohols and Glucosides) After two weeks Incubation at 35° C. (Continued).

Culture No.	Levu-lose	Malt-ose	Man-nose	Man-nitol	Melezi-tose	Raffi-nose	Rham-nose
H-138	0.6	0.4*	0.9*	0.9*	0.5	0.4	0.9*
H-238	0.6	0.1	1.0*	0.5	0.6	0.4	0.5
H-338	0.7	0.4	0.5	0.6	0.6	0.4	0.5
H-438	0.7	0.7	0.6	0.5	0.6	0.6	0.5
H-538	0.6	0.5	0.5	0.4	0.6	0.4	0.5
H-638	0.6	0.7	0.6	0.6	0.7	0.6	0.6
H-738	0.7	0.5	0.6	0.5	0.7	0.6	0.7
H-838	0.8	0.2	0.6	0.6	0.5	0.6	0.7
H-1138	0.7	0.7	0.7	0.7	0.7	0.5	0.6
H-1238	0.7	0.6	0.7	0.7	0.7	0.5	0.6
H-1338	0.8	0.7	0.6	0.6	0.7	0.5	0.6
H-1438	0.7	0.7	0.7	0.7	0.7	0.4	0.6
H-1538	0.7	0.6	0.6	0.7	0.7	0.2	0.3
H-1638	0.7	0.7	0.6	0.7	0.7	0.6	0.6
H-1738	0.8	0.8	0.6	0.6	0.7	0.5	0.6
H-1838	0.7	0.8	0.6	0.6	0.6	0.6	0.6
H-1938	0.7	0.5	0.7	0.6	0.6	0.0	0.5*
H-2038	0.7	0.7	0.5	0.6	0.7	0.1	0.6
H-2138	0.7	0.6	0.6	0.6	0.7	0.2	0.6
H-2238	0.7	0.6	0.6	0.6	0.6	0.4	0.6
Uninoc. Controls	0.4*	0.4*	0.3*	0.3*	0.3*	0.3*	0.3*

See footnote at end of table.

Table 13. Acid Production from Various Carbon Compounds (Carbohydrates, Alcohols and Glucosides) After two weeks Incubation at 35° C. (Continued).

Culture No.	Saccha-rose	Sala-cin	Sorbi-tol	Starch	Xylose	Basal Broth Control
H-138	0.4*	0.5*	0.0	0.4*	0.8*	0.8
H-238	0.6	0.7*	0.4*	0.2*	0.7*	0.7
H-338	0.8	0.5*	0.0	0.5*	0.2*	0.8
H-438	0.0	0.3	0.5	0.3*	0.0	0.8
H-538	0.2	0.3	0.5	0.4*	0.1*	0.8
H-638	0.7	0.3	0.1	0.5*	0.2*	0.8
H-738	0.1*	0.0	0.0	0.5*	0.1*	0.8
H-838	0.7	0.0	0.1	0.8*	0.0	0.8
H-1138	0.8	0.1	0.2	0.5*	0.2	0.7
H-1238	0.7	0.1*	0.1	0.7*	0.2*	0.8
H-1338	0.0	0.1	0.1	0.2	0.1*	0.8
H-1438	0.7	0.5*	0.3	0.2	0.3*	1.0
H-1538	0.2	0.5*	0.2*	0.6*	0.2*	0.8
H-1638	0.1*	0.2	0.1	0.4*	0.1	1.0
H-1738	0.0	0.1	0.1	0.4*	0.2*	0.8
H-1838	0.1	0.2	0.5	0.5*	0.0	1.0
H-1938	0.8	0.5*	0.1	0.6*	0.2*	0.8
H-2038	0.0	0.1	0.1	0.0	...	0.8
H-2138	0.0	0.3*	0.1	0.4*	0.0	0.8
H-2238	0.0	0.1*	0.4	0.5*	0.1	0.8
Uninoc. Controls	0.4*	0.3*	0.3*	0.4*	0.4*	0.4*

* Amount in cc. of N/10 NaOH required to neutralize a 10 cc. sample. Balance of the figures show amount in cc. of N/10 HCl required to neutralize a 10 cc. sample.

Table 14. Acid Production from the Fermentation of Dextrose Broth After four days Incubation at 35° C.

Medium: 0.5 per cent dextrose, 0.5 per cent K_2HPO_4, tryptone and peptone 0.5 per cent each.

Methods: Two cc. aliquots diluted with 35 cc. of distilled water, brought to a boil to expel CO_2, cooled and titrated with 0.111 N NaOH, using phenolphthalein as the indicator; values obtained expressed as grams lactic per 100 cc. of medium. pH determinations by the glass electrode.

Culture No.	Final pH	Grams lactic per 100 cc.		Culture No.	Final pH	Grams lactic per 100 cc.	
		I	C			I	C
Broth Control	7.38	0.120		H-1738	7.22	0.120	0.000
H-138	6.38	.300	0.180	H-1838	7.10	.130	.010
H-238	5.92	.360	.240	H-1938	7.25	.080	-.040
H-338	7.60	.070	-.050	H-2038	7.32	.100	-.020
H-438	7.52	.070	-.050	H-2138	7.35	.120	.000
H-538	7.52	.090	-.030	H-2238*	7.02	.130	.010
H-638	7.40	.080	-.040	H-139	7.02	.165	.045
H-738	7.38	.080	-.040	H-239	7.48	.070	-.050
H-838	7.38	.080	-.040	H-339	6.85	.180	.060
H-1138	6.92	.140	.020	H-439	7.36	.100	-.020
H-1238	6.96	.125	.005	H-539	7.02	.150	.030
H-1338	7.58	.070	-.050	H-639	7.12	.130	.010
H-1438	7.28	.120	.000	H-739	7.58	.070	-.050
H-1538	7.45	.150	.030	H-839	7.08	.150	.030
H-1638	7.48	.100	-.020	H-939	7.46	.075	-.045

I, Values include acid of broth control.
C, Corrected values.
*, Cultures through H-2238 from 1938 series, remaining cultures from 1939 series.

Table 15. Fermentation End-Products by Strains H-1438 (type strain) and H-138 (varient) from Dextrose Broth After four days Incubation at 35° C.

Medium: Fermentations were carried out in one liter flasks containing 400 cc. of medium with the following ingredients per 100 cc; 1.0 g. of dextrose, 0.5 g. of K_2HPO_4, 0.2 g. of yeast extract, 0.5 g. of tryptone.

Methods: Titratable acidity by the method described in Table 14. Volatile acids and esters according to the A.O.A.C. (3); Ethyl and Butyl alcohols by the method of Stahly, Osborn and Werkman (35); Gases evolved collected over saturated acidified brine solution and analyzed according to method described by Veldhuis and Etchells (39); pH determinations by the glass electrode.

Strain	Products in grams per 100 cc. of medium						
	Titratable acidity as lactic	Volatile acids as acetic	Alcohols		Esters as ethyl acetate	Gases	
			ethyl	butyl		CO_2	H_2
H-1438	-0.065*	a	0.113	0	0.03**	0.388	0.007
H-138	0.268*	0.027	0.073	0	0.02	0.159	0.004

* Values corrected for acid (0.102 g./100) present initially in the medium.

** Values corrected for blank (0.005 g./100) run on the medium.

a Absent, distillate alkaline.

N.B. Initial pH of the medium was 7.18 after four days of fermentation by strains H-1438 and H-138 the values were 7.68 and 5.38 respectively.

PRELIMINARY BIOCHEMICAL STUDIES

In the present investigation a study of the amount and composition of gases evolved from fermentations has been made by employing quantitative methods of analyses. Fermentations resulting from media containing various carbon sources, such as carbohydrates, alcohols and glucosides have been examined with respect to the components of the gases evolved. During the fermentation of dextrose, several of the factors influencing the fermentation proper have been investigated. This constitutes the principal work involved although other investigational phases are included.

Preliminary Experiments

A considerable amount of preliminary work was carried out relative to the preparation and methods of handling of a suitable apparatus for gas collection, since specific requirements were necessary. In addition, a number of preliminary fermentations testing the apparatus were conducted. Two sets of experimental fermentations are all that will be reported.

A drawing giving the specifications of the gas collection outfit designed for the studies is shown in Figure 4. Prior to using, it was sterilized at 15 pounds pressure for 10 minutes with an empty flask attached to protect the lower part of U-tube F. A small cotton plug was placed well up into the U-tube F that separates the culture medium from the collection flask A prior to autoclaving. The sterile outfit was handled as follows:

A sterile 50 cc. round bottomed flask containing the culture me-

GAS COLLECTION OUTFIT

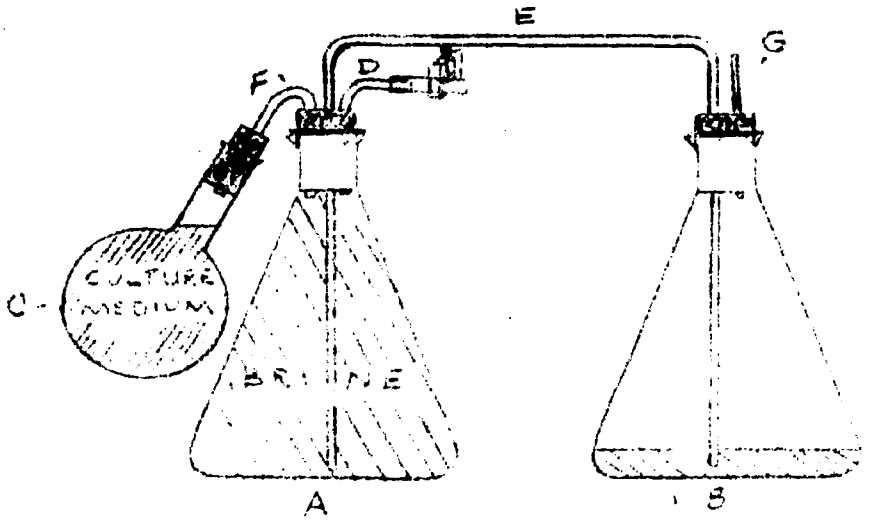

Fig. 4. The outfit consists of a one 50 cc. and two 250 cc. flasks. Flask A fitted with a three hole, No. 5 rubber stopper which is supplied with a short right angle tube D for gas outlet; a short U-tube F for attaching the 50 cc. round bottom culture flask C by means of a No. 0 rubber stopper; a long U-tube E for connecting flasks A and B. Flask B fitted with a two hole No. 5 rubber stopper through which passes the U-tube from flask A and an air outlet tube G. A short piece of rubber tubing is attached to tube D and supplied with a screw clamp when fermentation is started.

dium was inoculated and substituted for the empty flask. Saturated, acidified brine solution was placed in flask B and was brought up to the bottom of the rubber stopper in flask A through U-tube E and up into the gas outlet tube D by suction on a short piece of rubber tubing attached to D. The gas outlet was then sealed with a screw clamp. Incubation was at 35° C. Figure 5 shows an outfit prepared for incubation. One of the chief advantages of this type of apparatus, other than its simple construction and ease of handling, is that because of its small size and compactness a number of individual fermentations can be carried out at the same time in a limited amount of incubation space.

Treatment of Culture Flasks

Several gas collection outfits were constructed, tested and found free from leakage. Next to be considered was the development of a satisfactory method of handling the culture flasks in order to have the maximum amount of medium present and thus lessen the amount of air introduced into the produced gas. After several methods were tried, it was found possible to sterilize as much as 50 cc. of medium in the 50 cc. culture flask without excessive boiling or evaporation under any one of the following conditions: (a) when the flasks were plugged with cotton; (b) when the flasks were plugged with rubber stoppers supplied with short U-tubes plugged with cotton; (c) when the flasks were attached to the gas collection outfits.

However, preliminary experiments dealing with the amount of medium that could be used in the fermentations showed that approximately 57-58 cc. could be used in the culture flask with little or no difficulty arising from frothing during fermentation. This volume of medium brought the liquid level well up into the neck of the culture flask, leaving ap-

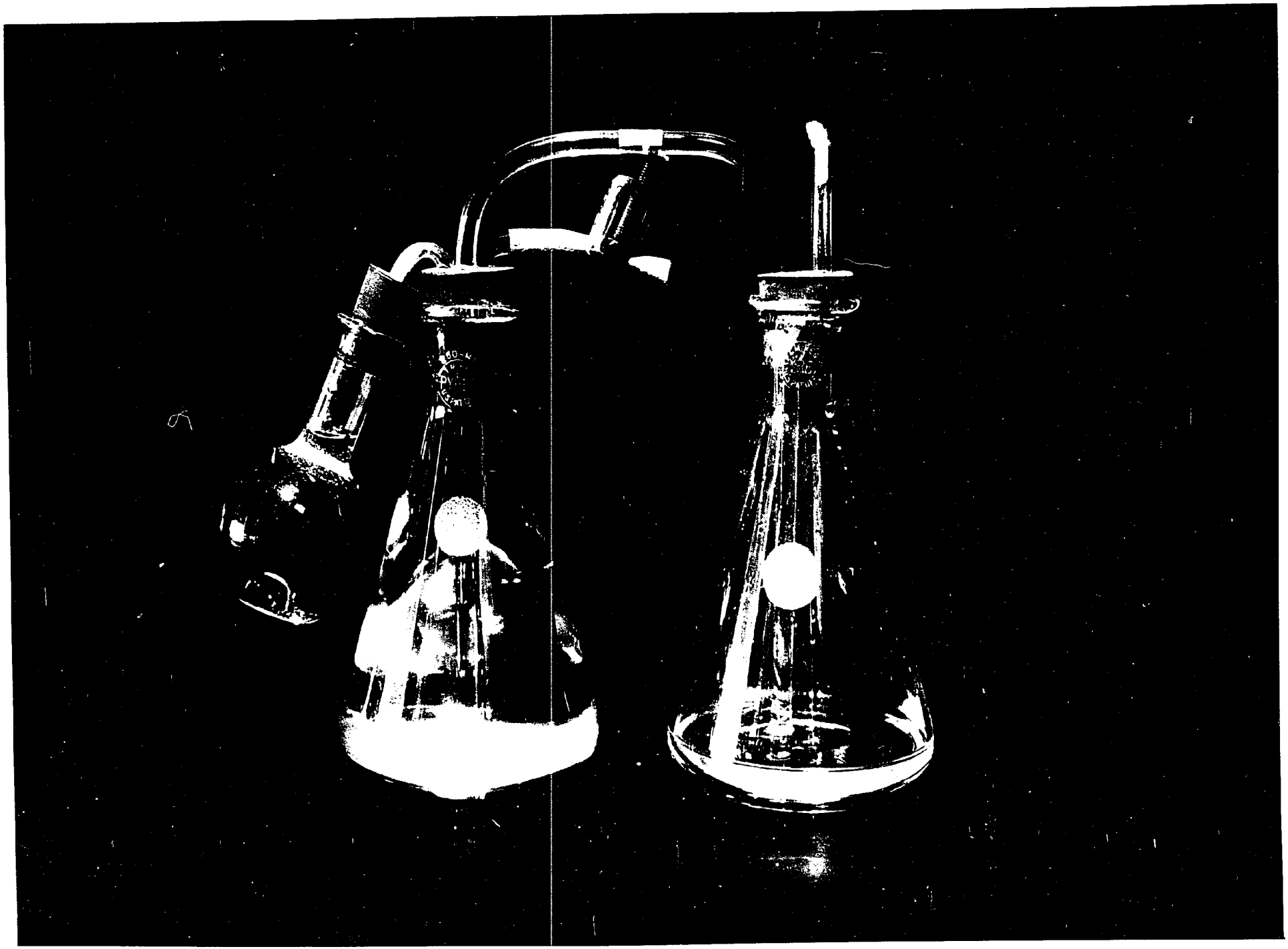

Fig. 5. Showing gas collection outfit at the start of fermentation.

proximately three to four cc. volume of air space (see Table 16) which would have little effect on the analysis of a 100 cc. volume of produced gas. It was necessary to aseptically pipette the additional seven to eight cc. of medium to the originally sterilized 50 cc. of culture medium in the fermentation flask.

The above treatment of culture flasks was used in the preliminary fermentations reported, however the procedure was slightly modified for the routine studies, one step being eliminated. The final procedure was as follows: the solutions to be tested were transferred to 125 cc. flasks, heated to 80° C. in a water bath, then sterilized by autoclaving at 10 pounds pressure for 10 minutes. Where compounds were used that were susceptible to heat, sterilization was carried out by Seitz filtration. The sterilized solutions were then poured aseptically to the graduated mark on the neck of the sterile 50 cc. calibrated culture flasks. After inoculation the flasks were placed on the gas collection outfits. Saturated, acidified brine solution was added to the receiving flasks and after the outfits were observed for one hour for leakage they were placed in the incubator.

Methods of Analyses

The methods of analyses for the gases produced were essentially the same for the preliminary studies as in the later more detailed investigations and will be outlined in detail at this time. Any variations from these methods will be noted in connection with the studies where they occurred.

The gas was measured and analyzed in a modified Williams gas analysis outfit, Model B. Carbon dioxide was determined by absorption in a

40 per cent solution of potassium hydroxide. Oxygen was determined by absorption in alkaline pyrogallol solution. Hydrogen was determined by firing with added oxygen in an electric spark explosion burette. Methane or other combustible hydrocarbons were determined by passing the products from the hydrogen determination through potassium hydroxide and noting the decrease in volume. This determination was always negative and is not reported. When possible, a 100 cc. volume of gas was analyzed as the apparatus was designed for maximum efficiency at this volume. Analyses of small volumes of gas (10 to 20 cc.) introduced a considerable error, brought about principally by: (a) insufficient amount of gas available so that a portion can be used for washing out the manifold and (b) difficulty in manipulation of the small amount of gas through the absorption solutions so as to obtain adequate contact. Residual (dissolved) gas remaining in the medium was determined by boiling the culture flask and trapping the gas driven off. In some instances this gas was analyzed separately while in others it was incorporated in the collected gas and the total gas analyzed. This method of determining the amount of residual gas was not wholly accurate since a small amount of gas was directly above the liquid, however, it gave a reasonable indication of the volume of the dissolved gas. Also, the analyses of the residual gas as to components are not comparable in accuracy to gas analyses run on larger volumes.

It should be noted at this time that in the gas analyses, the oxygen values were always found to be below the amount known to have been initially present at the start of fermentation. That is, if the oxygen value was multiplied by four to give the estimated nitrogen, and the sum

of the two volumes considered to be air, the figure did not total to the amount of air present above the culture medium at the start of fermentation. This would indicate that some of the oxygen was utilized by the fermentation as well as possibly being dissolved in the liquid system. This condition, especially in analyses of gas from active fermentations, favors a lower value of gas accounted for in the analyses since the remaining gas was calculated as nitrogen and this figure was calculated from the oxygen found present. Usually, the efficiency of the gas analyses ranged from 95-99 per cent.

Analyses of Gas from Cucumber Juice and Dextrose Broths

The first preliminary experiment was carried out to determine the amount and composition of the gases produced from fermenting cucumber juice broth. The juice had been previously prepared and was on hand in sterile 200 cc. amounts and was kept in 12 oz. brown "stubby" beer bottles supplied with crown closures. The medium was adjusted to pH 7.2* (see Table 17) heated, filtered and finally sterilized in 50 cc. amounts in culture flasks at 15 pounds pressure for 10 minutes. After cooling, the volumes were brought to 57-58 cc., the additions being made with a sterile pipette. The flasks were inoculated with one drop of an emulsion from an old agar slant of strain H-1438. The inoculated flasks were tightly fitted to the gas collection outfits after having first removed the auxillary flasks. (See Fig. 4) All outfits were supplied with brine solution and incubated at 35° C.

The second experiment was conducted to determine not only the amount and composition of gas resulting from the fermentation of dextrose

* pH determined with a glass electrode.

but also the rate of gas evolution. Tryptone (0.5 per cent) and yeast extract (0.2 per cent) were used as the basal medium to which 1.0 per cent dextrose was added. The broth was prepared in 100 cc. amounts and adjusted to the different pH values by use of sodium hydroxide and hydrochloric acid (see Table 18). Duplicate sets were adjusted to the following initial pH values 2.75, 5.05, 6.80 and 9.05. The adjusted broth was then added to sterile 50 cc. culture flasks in 50 cc. amounts and sterilized at 15 pounds for 10 minutes. After cooling, the final volumes were brought to 57-58 cc. by the addition of more broth by a pipette. All sets were inoculated with one drop of a 24 hour broth culture of strain H-1438 and the flasks were handled in the manner previously described for the gas collection outfits. Incubation was at 35° C.

During the fermentation period the brine level in the gas receiving flask A (see Figure 3) was marked daily and at the conclusion of the experiment the rate of gas production was determined by brine displacement.

Results

The result of the fermentations of cucumber juice broth as to gas evolution and composition are shown in Table 19. The mean values of five determinations show that this medium yielded gas composed of 81.0 per cent carbon dioxide and 16.4 per cent hydrogen. The ratio of hydrogen to carbon dioxide was 1:5. There were variations between individual fermentations although not sufficient to be considered significant. The analyses of the residual gas are shown in Table 20 and consisted principally of carbon dioxide (83.0 per cent).

Table 21 gives the results of the fermentations of dextrose broth with respect to gas production and composition. The percentages of carbon dioxide and hydrogen were found to be similar regardless of the initial pH adjustment. No significant differences were noted that were not exceeded by variations within the duplicates. The ratio of hydrogen to carbon dioxide for the three lots at pH 5.05, 6.80 and 9.05 were: 1:2.1; 1:2.3 and 1:2.3 respectively. There is practically no difference between the three ratios. However, they do show a considerable increase in proportion of hydrogen when compared with cucumber juice broth fermentation which was 1:5.

The gas production for the above series was recorded at intervals up to 14 days and the results are shown in Table 22. This material is presented graphically in Figure 6. It will be seen that the fermentation in all lots was rapid, with little gas being produced after three to four days' incubation. The fermentation in the unadjusted lot (pH 6.8) was the most rapid and showed some increase in gas production compared with the other lots adjusted to pH 5.05 and 9.05.

The most important results to be noted from these preliminary experiments were: (a) a satisfactory apparatus for studying individual fermentations was devised and tested; (b) the gases produced from cucumber juice and dextrose proved to be composed solely of hydrogen and carbon dioxide from fermentations vary, depending upon the carbon source fermented; (d) the fermentations take place over a considerable range with respect to initial pH adjustment and (e) during fermentation the gas production was rapid, the major portion being produced within about 48 to 72 hours.

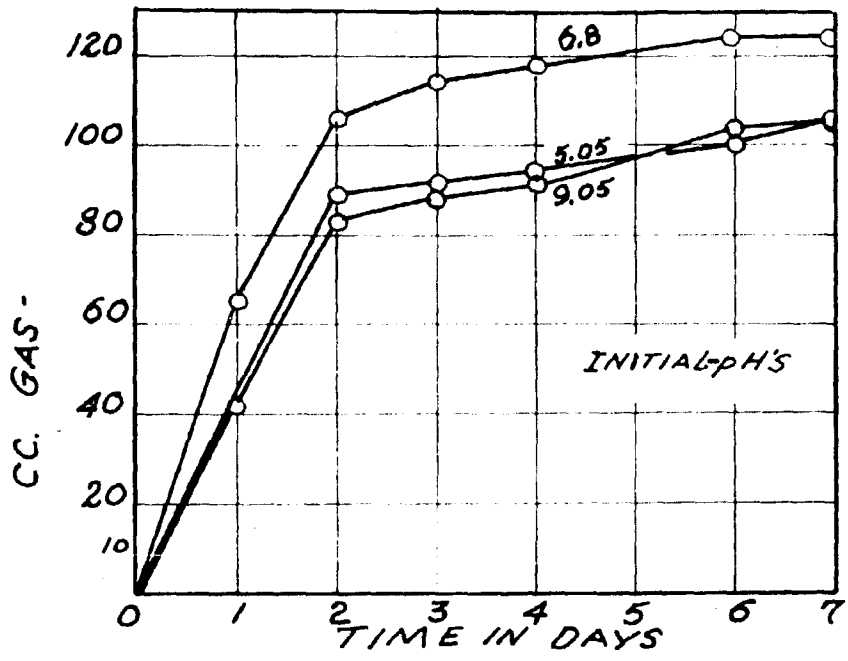

Fig. 6. Effect of initial pH adjustment of the culture medium upon gas evolution from the fermentation of dextrose.

Table 16. Calibration of Gas Collection Outfits.

Culture flask No.	Liquid volume of culture flasks to graduated mark*	Factor **	Volume of air from liquid surface to rubber stopper	Volume of delivery tube from culture flask to gas collection flask
	cc.		cc.	cc.
1	56.5	.884	2.0	1.2
2	57.0	.875	2.5	1.3
3	57.5	.867	2.5	1.2
4	57.0	.877	2.8	1.2
5	56.0	.892	2.8	1.2
6	58.0	.862	3.2	1.3
7	57.0	.875	3.0	1.2
8	55.5	.900	2.5	1.2
9	56.0	.892	2.5	1.2
10	58.0	.862	3.0	1.2
11	57.0	.875	2.5	1.2

* Culture flasks filled to a mark on the neck at a point leaving a minimum of air space and yet allowing for some foaming.

** The factor given in column three is determined by dividing 50 by the culture flask capacity shown in column two. This was done to place all gas analysis results on a comparable basis.

Table 17. Amount of Acid or Alkali Necessary to Adjust 100 cc. of Cucumber Juice Broth and Diluted Cucumber Juice Broth Aseptically to Desired pH Range.

Amount of N 1 HCl	pH *	pH **	Amount of N 1 NaOH	pH *	pH **
cc.t			cc.t		
10.0	1.9	...	0.0	5.2	5.3
8.0	2.5	...	0.2	5.4	5.7
6.0	3.0	2.0	0.4	5.6	6.4
5.0	3.4	2.3	0.6	5.8	7.1
4.0	3.6	2.5	0.8	6.3	8.1
3.6	...	2.7	1.0	6.7	8.9
3.2	3.8	3.0	1.2	7.1	9.3
2.8	4.0	3.2	1.4	7.4	9.6
2.4	4.1	3.2	1.6	7.8	9.9
2.0	4.3	3.5	1.8	8.4	10.1
1.6	4.5	3.7	2.0	8.7	
1.2	4.6	4.0	2.2	9.1	
0.8	4.9	4.3	2.4	9.2	
0.4	5.1	4.7	2.6	9.4	
0.2	5.2	5.0	2.8	9.6	
0.0	5.3	5.3	3.0	9.8	

* Cucumber juice broth.

** Cucumber juice broth diluted with equal parts of distilled water.

t These figures based on original calculations on 5 cc. amounts of broth.

Table 18. Amount of Acid or Alkali Necessary to Adjust Aseptically Tryptone-yeast Extract Broth in 5 cc. and 100 cc. Amounts to Desired pH Range.

Amount of N 1/10 HCl for 5 cc. of broth	pH	Amount of N 1 HCl for 100 cc. of broth	pH
cc.		cc.	
1.4	2.7	2.8	2.9
1.2	2.9	2.4	3.0
1.0	3.2	2.0	3.2
0.8	3.5	1.6	3.4
0.6	3.9	1.2	3.8
0.4	4.8	0.8	4.4
0.2	5.4	0.4	5.3
0.0	7.0	0.2	6.0
		0.0	7.0
Amount of N 1/10 NaOH		Amount of N 1 NaOH	
0.0	7.0	0.0	7.0
0.2	8.3	0.2	7.7
0.4	9.1	0.4	8.3
0.6	9.5	0.8	9.1
0.8	9.9	1.2	9.5
1.0	10.3	1.6	10.0
		2.0	10.4

Table 19. Analyses of Gas Produced from the Fermentation of Cucumber Juice Broth (pH 7.2) by Organism No. H-1438

Flask No.	Vol. of collected gas	Carbon Dioxide		Hydrogen		Oxygen		Remainder gas*	Ratio of H_2:CO_2**
	cc.	%	cc.	%	cc.	%	cc.	cc.	
1	136	82.2	112	14.1	19	0.3	0.4	5	1:5.8
2	214	78.0	167	19.6	42	0.4	0.9	4	1:4.0
3	170	80.9	137	15.4	26	0.3	0.6	6	1:5.2
4	200	81.8	164	15.6	31	0.4	0.8	4	1:5.2
5	210	82.0	172	17.4	37	0.2	0.4	4	1:4.7
Mean	186	81.0	150	16.4	31	0.3	0.6	5	1:5.0

* Principally nitrogen from the air initially present above culture medium.

** Calculated from the hydrogen and carbon dioxide percentages.

N.B. Analysis of the cucumber juice showed 1.92 grams of reducing sugars per 100 cc.

Table 20. Analyses of Residual Gas from Culture Medium of Fermentations Shown in Table 19.

Flask No.	Vol. of Residual gas*	Carbon dioxide		Hydrogen		Remainder gas
	cc.	%	cc.	%	cc.	cc.
1	21	86.1	18	7.0	1.5	1.5
2	14	80.5	11	6.2	0.9	2.1
3	18	85.5	15	8.7	1.6	1.4
4	24	84.7	20	8.4	2.0	2.0
5	14	78.5	11	12.2	1.7	1.3
Mean	18	83.0	15	8.5	1.5	1.6

* Gas dissolved in culture medium and present above culture medium.

Table 21. Analyses of Gas Produced from the Fermentation of One per cent Dextrose at Different Initial pH Values by Organism No. H-1438; Basal Broth of Tryptone and Yeast Extract.

Flask No.	Amount of broth	Vol. of collected gas	pH	Carbon dioxide		Hydrogen		Oxygen		Remainder gas*	Ratio of H_2:CO_2**
	cc.	cc.		%	cc.	%	cc.	%	cc.	cc.	
10	58.0	125	5.05	65.6	82	30.5	38	0.4	0.5	4	1:2.2
7	57.5	124	5.05	60.3	75	34.8	43	0.4	0.5	5	1:2.0
Mean	57.8	125	5.05	63.0	79	32.7	41	0.4	0.5	5	1:2.1
2	56.5	138	6.80	66.8	92	29.4	39	0.3	0.4	7	1:2.3
1	58.5	147	6.80	65.7	97	29.5	43	0.3	0.4	7	1:2.2
Mean	57.0	143	6.80	66.3	95	29.5	41	0.3	0.4	7	1:2.3
4	57.0	114	9.05	65.7	75	29.4	34	0.3	0.3	5	1:2.4
3	58.0	130	9.05	66.8	87	29.4	38	0.3	0.4	5	1:2.2
Mean	57.5	122	9.05	66.3	81	29.4	36	0.3	0.4	5	1:2.3
5	58.0	0	2.75	0	0	0	0	0	0	0	0
9	58.0	0	2.75	0	0	0	0	0	0	0	0
Mean	58.0	0	2.75	0	0	0	0	0	0	0	0

* Principally nitrogen from the air initially present above culture medium.
** Calculated from the hydrogen and carbon dioxide percentages.

Table 22. Amounts of Gas Produced by Organism No. H-1438 from Dextrose Broth (1%) Adjusted to Different Initial pH Values.

Flask No.	Amount of broth in cc.	pH	Total vol. of gas collected in cc. by days							
			1	2	3	4	6	7	8	14**
10	58.0	5.05	68	130	130	130	130	130	130	130
7	57.5	5.05	30	75	81	86	100	111	122	122
Mean	57.7	5.05	49 (42)t	103 (89)	106 (92)	108 (94)	115 (100)	121 (105)	126 (109)	126 (109)
2	56.5	6.80	76	124	128	134	139	139	139	139
1	58.5	6.80	73	122	134	138	142	142	142	142
Mean	57.5	6.80	75 (65)	123 (106)	131 (114)	136 (118)	141 (122)	141 (122)	141 (122)	141 (122)
4	57.0	9.05	44	91	96	96	114	114	114	114
3	58.0	9.05	52	100	106	118	126	126	126	126
Mean	57.5	9.05	48 (42)	96 (83)	101 (88)	107 (93)	120 (104)	120 (104)	120 (104)	120 (104)

* Basal medium of tryptone (0.5%) and yeast extract (0.2%).
** Total volume of gas collected, after 14 days fermentation.
t Figures in parentheses calculated on basis of gas evolved from 50 cc. of culture medium.

PRINCIPAL BIOCHEMICAL STUDIES

The preliminary investigations were followed with more extensive fermentation studies with respect to gas production, rate of gas production and composition of the evolved gases. More specifically, the experimental work, in relation to the above general grouping, covered the following phases: (a) Fermentations by different strains; (b) fermentations by the same strain; the effect of (c) temperature, (d) pH and, (e) salt* upon the fermentation of dextrose; (f) the effect of heat upon the culture medium; (g) the effect of dextrose on the fermentation of maltose; (h) the fermentation of various carbon compounds. Experiments somewhat unrelated to the above fermentation series dealing with (i) the effect of heat on the viability of two strains are included. The experimental work will be presented in the order listed above in Tables 23 to 38.

Procedure

The gas collection outfits, methods of gas analyses, determination of residual gas, determination of the rate of gas evolution and the preparation of the test carbon compounds have all been previously described.

General procedure not previously described will be presented in part at this time; the balance will be given when the individual experiments under consideration are taken up.

The basal broth to which the fermentable carbon compounds were added was modified slightly (from the original description) to exclude yeast extract, since this material had a tendency to promote frothing in the neck of the culture flasks during fermentation. The final basal broth consisted solely of tryptone (0.5 per cent), which was found satisfactory

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* The effect of salt upon the fermentation of cucumber juice is also included.

for the growth requirements of the organisms.

For the fermentations carried out in buffered broth, the procedure was as follows: A stock buffer solution (33) was prepared containing 20 g. of ammonium dihydrogen phosphate, 45 g. of dipotassium hydrogen phosphate and 71 g. of citric acid per liter. One hundred cc. quantities of this buffer solution were adjusted to the desired pH values with 10 N sodium hydroxide (Figure 7). Ten cc. of the adjusted stock solutions per 100 cc. of basal medium were usually sufficient to obtain the required buffering effect. In experiments involving the use of a liter or more of culture medium, it was found practicable to use one-tenth of the amounts of the chemicals listed above in one liter of the basal broth and then adjust this amount with 1 N sodium hydroxide to the desired pH. This procedure was used to obtain the buffered broth (pH 5.15) used in a number of the experiments.

All gas volumes reported were calculated on the basis of gas resulting from 50 cc. of culture medium containing 1.0 per cent of the carbon compounds*, which would be in terms 0.5 g. of fermentable carbon source present**. Actually, the fermentation culture flasks held from 55.5 to 58.0 cc. (see Table 16), hence such a calculation is in keeping with the actual amount of fermentable carbon source tested. The conversion factors used in these calculations are shown in Table 16. The hydrogen and carbon dioxide ratios, in all cases, are based on the actual

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* With the exception of lactose, raffinose, rhamnose and salacin (Table 36), here the amount was increased to 3.0 per cent to obtain sufficient gas for analysis.

** Where cucumber juice was employed as the nutrient medium, the calculations were based on gas production from 50 cc. of 2.0 per cent carbohydrate (equal to 1.0 g. of CHO present).

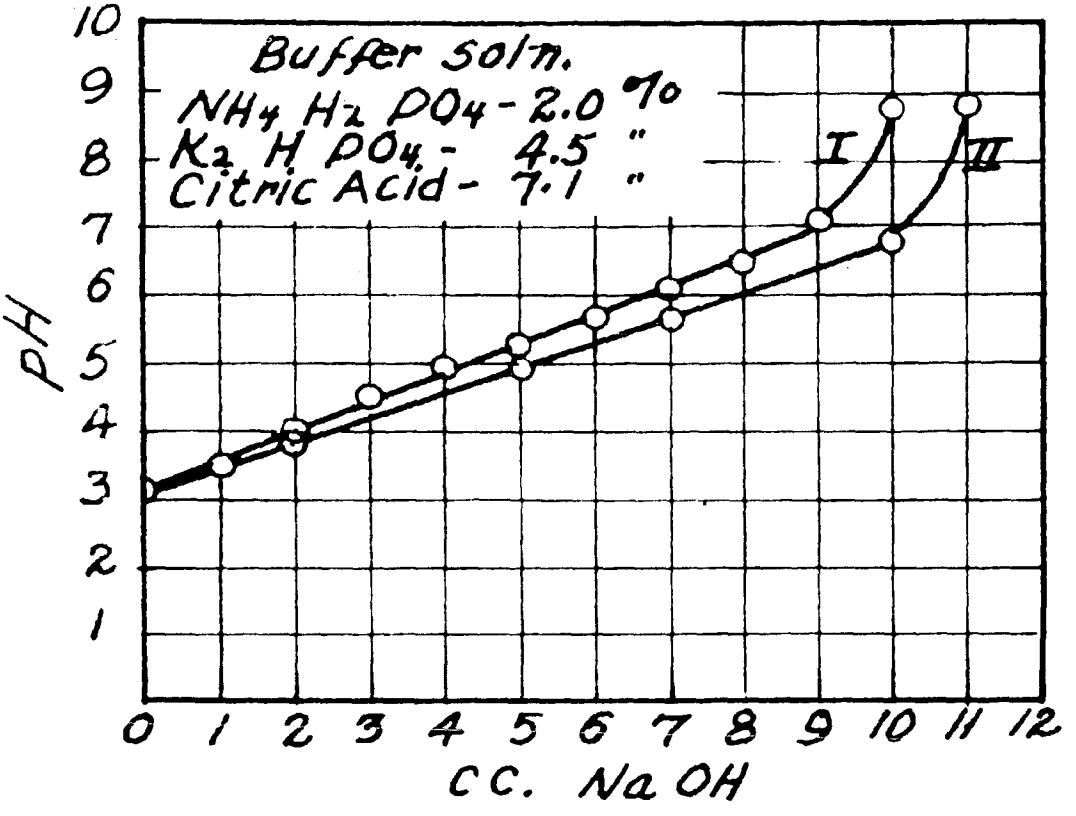

Fig. 7. pH change in buffer solution upon the addition of NaOH. I, 10 cc. of buffer soln. plus 1N NaOH; II, 100 cc. of buffer soln. plus 10N NaOH.

volumes of these gases produced and include both evolved gas (also designated as collected gas) and residual gas.

In the major portion of the experiments, the gas analyzed represented an aliquot from the resulting fermentations and included the residual gas incorporated at the time of analyses. However, in two sets of experiments (d and h) residual gas was determined and analyzed separately.

All fermentations, with the exception of the temperature series (c), were conducted at 35° C. Gas volumes were corrected to room temperature (23° C. for these experiments). Corrections as to variations in barometric pressure were too small when applied to 100 cc. gas volumes to be considered significant. All inoculations were made from actively growing, 24 to 48 hour cultures, inoculation being made with one drop of culture added by a sterile pipette. Strain H-1438 was used in all experiments as the test organism with the exception of the comparative study upon fermentations brought about by several strains (a). In the viability experiments (i), two strains were used; H-1438 and H-138. All pH determinations were made with a glass electrode.

Results

a. Fermentations by different strains

Several strains were investigated with respect to production and composition of gas. The fermentations were carried out in buffered (pH 5.15) dextrose (1.0 per cent) broth containing 0.5 per cent tryptone. The following strains were used: H-739, H-439, H-639, (1939 series), H-638, H-1338, H-438 and H-138 (1938 series). Incubation was for one week at 35° C. The gas outfits were marked at 24 hour intervals for rate

of gas production. At the conclusion of the incubation period the gas in the different outfits was analyzed.

The results are shown in Tables 23 and 24. The data from Table 24 are presented graphically in Figure 8. The composition of gas was the same for all the fermentations with all the strains except strain H-138. The gas from these strains showed hydrogen to carbon dioxide ratios between 1:2.32 and 1:2.59. The maximum amount of gas produced by five of the above group also was quite similar, the range being from about 139 to 148 cc. However, in the case of strain H-138 a difference in behavior was demonstrated, not only by a decrease in the amount of gas produced but also by the proportion of hydrogen in the gas which was greater (1:1.44).

In general, it is noted (Figure 8) that the fermentation with all strains is rapid with the major portion of the gas evolved within 48 hours. In these fermentations, little or no gas was produced after four days. Also, the gas evolution curves show that five* of the seven strains tested produced gas volumes well above the 100 cc. range.

b. Fermentations by the same strain

The results of quadruplicate fermentations of dextrose broth by strain H-1438 are shown in Table 25; parts A and B show gas composition and rate of gas evolution respectively.

The percentages of carbon dioxide and hydrogen are comparable for all fermentations, the greatest variation observed being about two per cent for each component. The relationship of the gases is presented again in the hydrogen to carbon dioxide ratios. Here the variations between fermentations were from 1:2.35 to 1:2.49, such differences being

* Curve for H-438 inadvertently omitted; data in Table 24 indicates it would be practically identical with H-1338.

considered insignificant.

The data from Table 25, part B are reproduced in Figure 9. The fermentations were rapid; all producing well over 100 cc. of gas in 48 hours. Three of the fermentations resulted in practically the same amount of evolved gas; the fourth fell a little short of the amount attained by the others.

c. The effect of temperature on the fermentation of dextrose.

The influence of temperature with respect to gas production and composition is shown in Table 26. Eight temperatures, ranging from 5° C. to 45° C. were employed. Experimental procedure concerning the broth, inoculation and the strain used have been previously described. The lower and higher limitations for the fermentation yielding gas were found to be 5° and 45° C. No perceptible growth was noted at the lower limit, while at the higher limit, perceptible growth was observed but no gas detected, either evolved or residual. At 13° C. almost one-half of the produced gas was residual, due to the greater solubility of carbon dioxide at lower temperatures. The temperatures 19°, 24° and 40° C. were similar with respect to the total amount of gas present at the end of fermentation. It would appear that the optimum temperature for gas production lies within the 35° C. range. The composition of the gas from all fermentations (13° to 40° C.) was comparable as to percentages of hydrogen and carbon dioxide found; 28.1 to 33.5 H_2 and 64.6 to 69.2 CO_2 per cent respectively. The hydrogen and carbon dioxide ratios likewise show similar relationships for this series.

The effect of temperature on the gas evolved and rate of evolution is shown in Table 27 and Figure 10. Thirty-five degrees C. shows

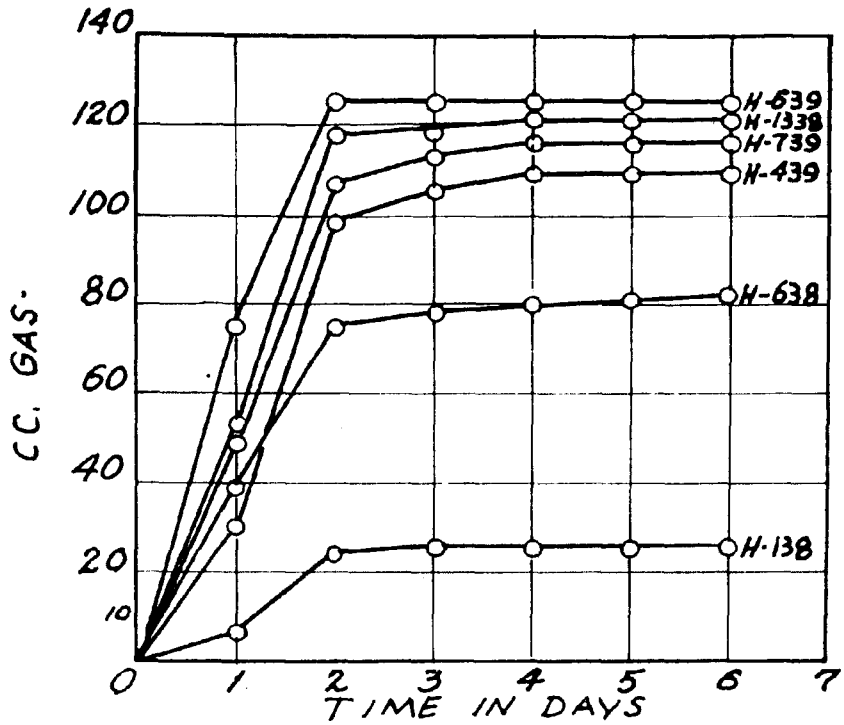

Fig. 8. Gas evolution from the fermentation of dextrose by several strains of the stock culture collection.

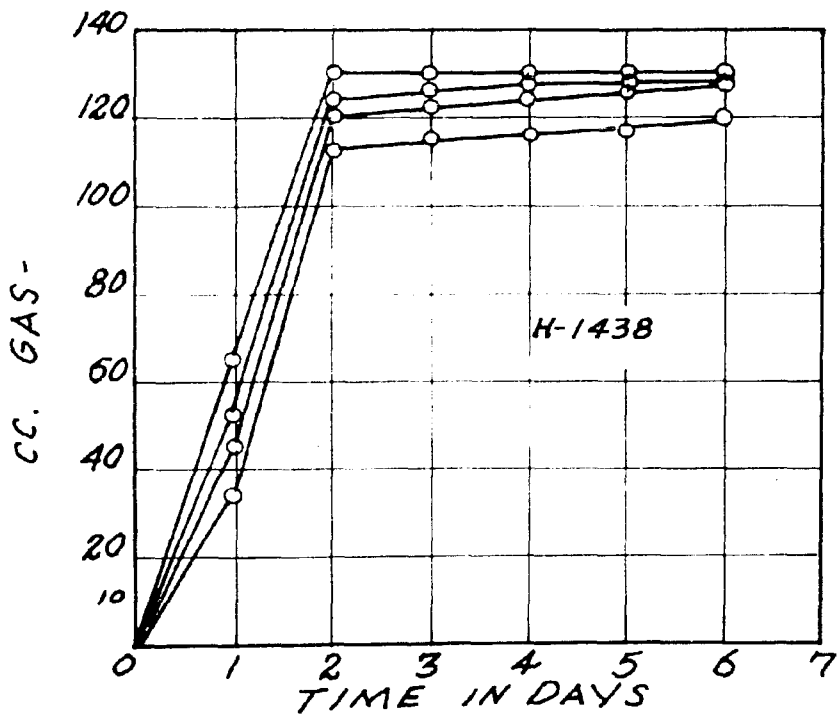

Fig. 9. Gas evolution from quadruplicate fermentations of dextrose by strain H-1438.

the most rapid evolution as well as the maximum amount of gas evolved. Fermentations either above (40° C.) or somewhat below (24° C.) the optimum (35° C.) were considerably retarded and less gas was evolved. Gas evolution at 19° C. was much slower than at 24° C., but at the end of the incubation period (eight days) the amount of gas evolved was about the same. At 13° C., the gas produced during the first three to four days was dissolved in the culture medium. However, it is evident that this temperature definitely retarded the fermentation.

d. The effect of pH on the fermentation of dextrose.

For this series, 100 cc. amounts of the stock buffer solution, prepared as previously described, were adjusted with 10 N sodium hydroxide to cover a range of eight pH values. Ten cc. amounts at each desired range were supplemented with 1.0 g. of dextrose and 0.5 g. of tryptone and made up to 100 cc. volume. Determinations with respect to pH were made before and after sterilizing. The final pH values for the different lots extended from 3.6 to 8.85. An unbuffered control (pH 6.8) was included.

The results show (Table 28) that the fermentation takes place over a considerable pH range. No growth resulted at pH 3.6 in the acid range while pH 8.85 seemed to approach the limit for adequate growth in the alkaline range; the latter being assumed on the basis of marked decrease in gas evolution at this pH. Of the pH values studied, 5.3 appears to be the optimum in the buffered series, although the unbuffered control (initial pH 6.8) resulted in a slightly greater volume of total gas produced. In general, it is shown from considering the hydrogen and carbon dioxide ratios that no great difference in gas composition existed,

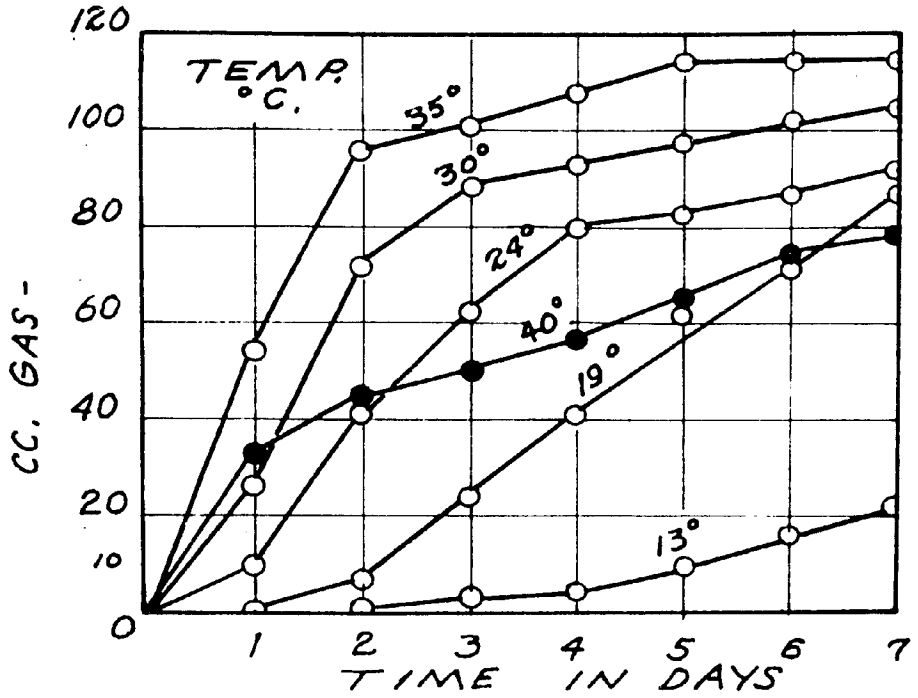

Fig. 10. Effect of temperature on gas evolution from the fermentation of dextrose by strain H-1438.

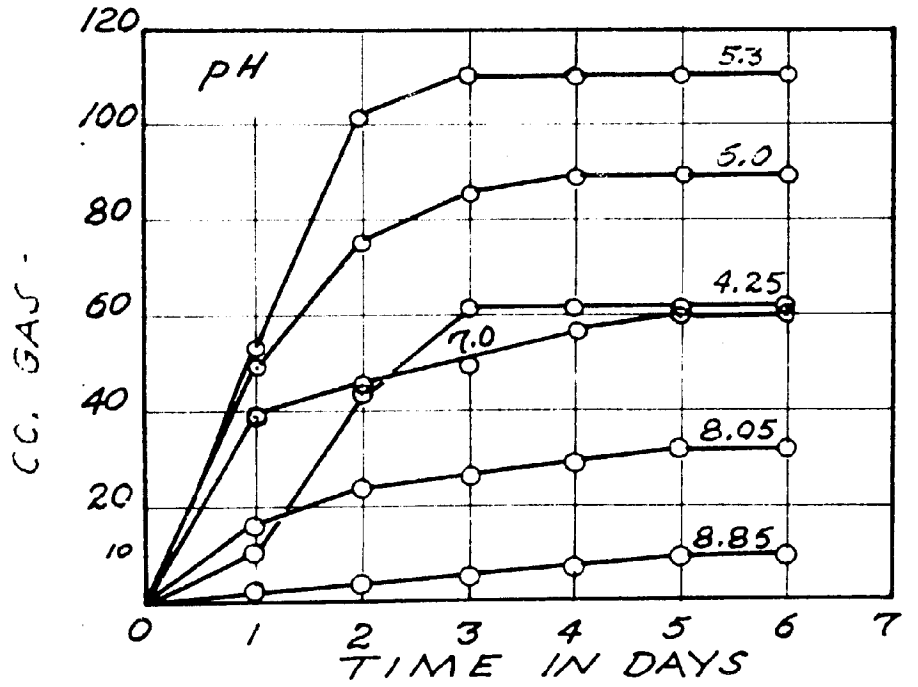

Fig. 11. Effect of buffered pH on gas evolution from the fermentation of dextrose by strain H-1438.

although there seems to be a slight increase in proportion of hydrogen from the fermentations where the pH range increases above 7.0; likewise, this seems probable for the lower value, namely, 4.25.

The analyses of the residual gas from the fermentations are presented in Table 29. The amount of residual gas increases with the more alkaline pH values, due to the greater solubility of carbon dioxide. The percentage of carbon dioxide was found to be about the same in all cases and this gas constituted the major portion of the total residual gas.

The rate of gas evolution for the buffered series is given in Table 30. These data (with the exception of pH 7.55*) are presented in Figure 11. A comparison of the gas evolution curves shows that the most rapid evolution of gas, as well as the greatest amount, resulted from the fermentation at pH 5.3. At pH 6.0 the rate of gas evolution was not particularly influenced although there was a decrease in the amount of gas evolved. Fermentations at pH 4.25, 7.55 (not shown) and 7.0 were comparable with respect to rate of gas evolution, the major portion of the gas being evolved within three to four days. However, only a little more than one-half the amount of gas was evolved from these fermentations as compared to the optimum (pH 5.3). The effect of the more alkaline pH values (8.05 and 8.85) was shown most clearly by a marked decrease in gas evolution.

e. The effect of salt (NaCl) on the fermentation of dextrose and cucumber juice.

Calculated amounts (Table 31) of C.P. salt were added to 100 cc.

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* Omitted for sake of clarity in presentation; followed 7.0 very closely.

volumetric flasks and made up to volume with 1.0 per cent dextrose broth buffered at pH 5.15; the salt concentrations employed were; 5, 10, 15, 20 and 25 per cent saturation with respect to salt. This series, after sterilization, was inoculated with strain H-1438 and incubated one week at 35° C. In all fermentations, the residual gas was boiled out and included in the collected gas at the time of the analyses.

The results for the dextrose lots are shown in Table 31, part A. There was a marked decrease in gas production as the salt concentration increased above 5 per cent saturation. Salt concentrations as high as 20 and 25 per cent saturated resulted in no growth. In all fermentations in this series (including the control) the percentages of hydrogen and carbon dioxide were similar with the possible exception of those from the fermentation at 15 per cent saturation. In the latter case, the proportion of hydrogen found was higher, however the volume of gas available for analysis was small (13.1 cc.) which influenced the accuracy of the determination.

It is of particular interest to note the effect of salt upon the buffered pH (Figure 12). In the dextrose lots, the pH values decreased below the optimum (5.3 to 5.15) with the addition of salt to the extent of 15 per cent saturation and decreased further with higher salt concentrations. This was not desirable since some inhibitory effect of the lower pH values was probably exerted upon the fermentations. Consequently, a similar experiment was set up using cucumber juice broth, previously adjusted to pH 5.9 so that the addition of salt would not bring the pH to the inhibitory range. The results are shown in Table 13, part B.

In this series it is evident that the salt had the same general effect upon gas production as previously experienced with the dextrose

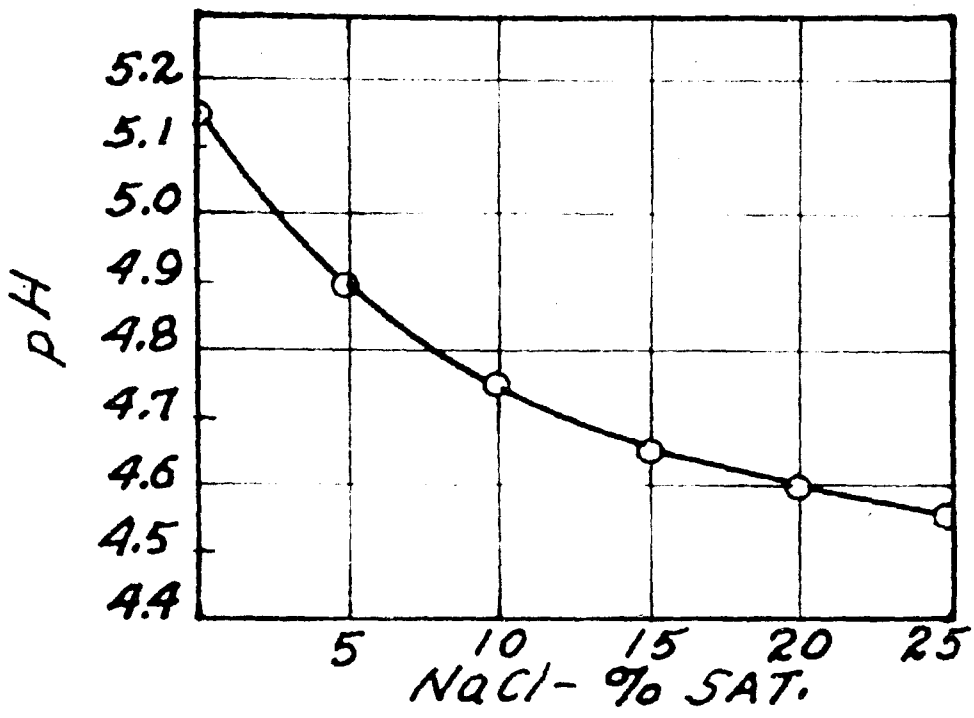

Fig. 12. Effect of NaCl upon the pH of dextrose broth buffered at pH 5.15.

lots (part A) Although adequate growth and gas production resulted in the 20 per cent saturated lot. Slight growth was noted in the 25 per cent saturated lot but evidently not sufficient for gas production. The small differences in gas composition are not considered particularly significant. The effect of salt on the pH of the cucumber juice broth was similar to that upon dextrose broth. However, since the initial adjusted pH of the former was higher, the drop due to salt did not bring the final values to the inhibitory range for the fermentations in the high salt concentrations.

Table 32 presents the results with respect to gas evolved and the rate of evolution for both the dextrose and cucumber juice lots. These data are shown graphically in Figure 13 and are calculated on the same basis (50 cc. of 1.0 per cent carbohydrate present) to facilitate comparison between the two lots of media used. It is evident that salt above five per cent saturation exerts a decided effect upon the fermentation which is demonstrated by a reduction in the gas evolved as well as a retardation in the rate of evolution. A part of the effect of salt in the dextrose series can be attributed to the influence exerted upon the pH of the medium.

f. The effect of heating the culture medium.

Upon several occasions, delayed fermentations of cucumber juice have been experienced. Usually, such fermentations were accompanied by a decrease in gas evolution. A check of the procedure that was followed in such instances revealed that the only variable factor was the length of time* the juice had been subjected to the usual steam pressure (15

* Time increases resulted chiefly through re-sterilization of previously opened, unused lots.

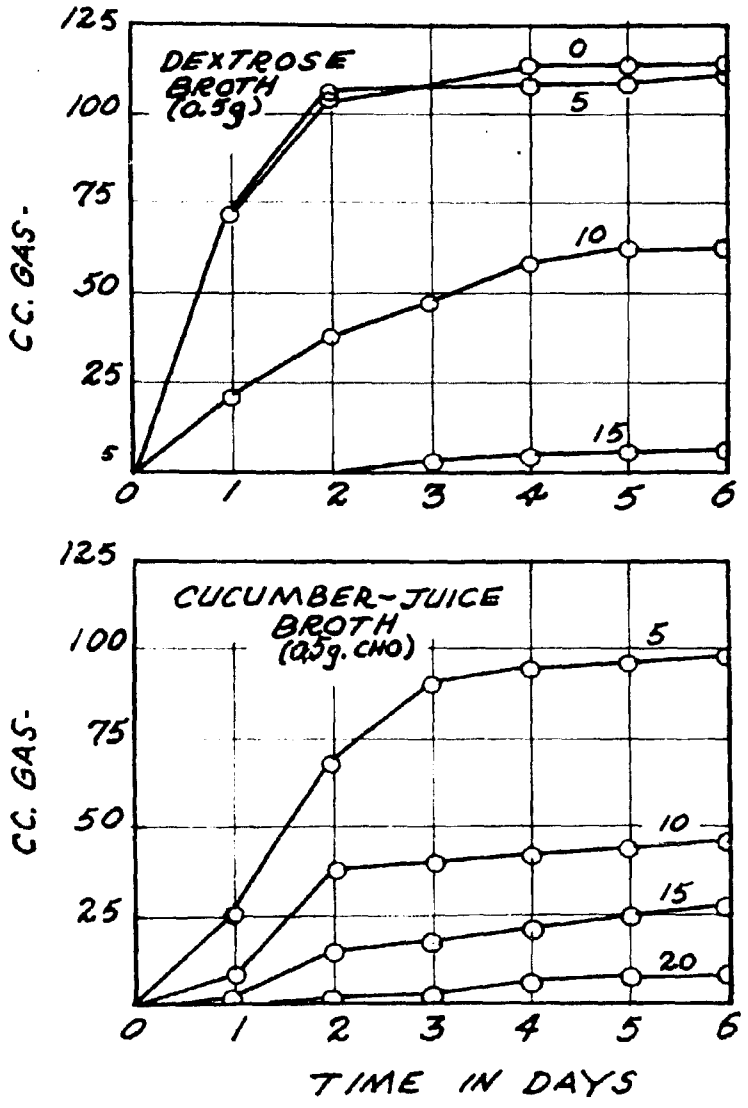

Fig. 13. Effect of NaCl upon gas evolution from the fermentation of dextrose (upper part) and cucumber juice (lower part). Curves identified according to per cent saturation of salt.

lb.) employed for sterilization.

Consequently, a study was made to determine the effect of heat upon cucumber juice as evidenced by subsequent fermentation with and without the presence of salt. In the salt studies, the chief aim lay in the possibility of bringing about a more vigorous fermentation in the presence of salt at one of the higher concentrations (15 per cent saturation), with which only moderate success had been previously (Table 32) obtained. Also, the possibility of the influence of plant salt (the grade used at commercial plants) as compared to C.P. salt was given attention.

Procedure

Fresh cucumbers were frozen and thawed to facilitate removing the juice and the latter was centrifuged one hour at 2,000 r.p.m. in 250 cc. amounts and sterilized by Seitz filtration. Fermentations were set up as follows: The first lot of Seitz filtered juice was divided into three parts; the first was unheated, the second was autoclaved 40 minutes at 15 lb. pressure and a third was autoclaved 100 minutes at the same pressure. These three lots of juice were labeled I, II and III respectively. A second lot of juice was given the above treatments and in addition was supplemented with salt to the extent of 15 per cent saturation. These three lots were labeled IV, VI and VII. Also, one portion of unheated juice was included in which the salt added was obtained from a commercial pickling plant which was listed as C grade mine salt. This was labeled V. The inoculations for the salt series were made from a 48 to 72 hour cucumber juice broth culture of strain H-1438 growing at the above salt concentration. Analyses were made after one week incubation. All analyses included residual gas incorporat-

ed into the collected gas at the time of analyses.

Results

The results (Table 33) show that of the fermentations resulting from the Seitz filtered juice (I, II and III), the unheated (I) juice gave a considerable increase with respect to total gas production. The difference between the 40 and 100 minute heated lots was not outstanding, although slightly less gas (about 20 cc.) resulted from the 100 minute treatment. The composition of the gas from all three treatments was similar; the hydrogen and carbon dioxide ratios for the lots of juice were as follows; I, 1:3.50; II, 1:3.97 and III, 1:4.08. The differences were well within the limits of fermentation differences. The fermentations in lots IV, V and VI show no particular difference with respect to total gas production. However, the 100 minute heating (VII) markedly decreased the amount of gas produced. The composition of the gas from this series, as indicated by the percentage of carbon dioxide found, shows no significant differences. However, it is evident that the usual correlation between the two gases was absent since their sum lacks considerable of totaling 100 per cent. This points toward an error in the determination of hydrogen, although the identity of the difficulty was not learned.

The rate of gas evolution for the fermentations with and without salt are presented in Table 34 and Figure 14. Results from treatment V, (plant salt) is omitted inasmuch as they were similar to IV. The results show a very striking relationship with regard to the speed of fermentation as influenced by heat in the salt-free treatments. At the 48 hour interval, the unheated juice (I) demonstrated a two-fold increase in gas evolution over the 40 minute heat treatment (II) and a twenty-

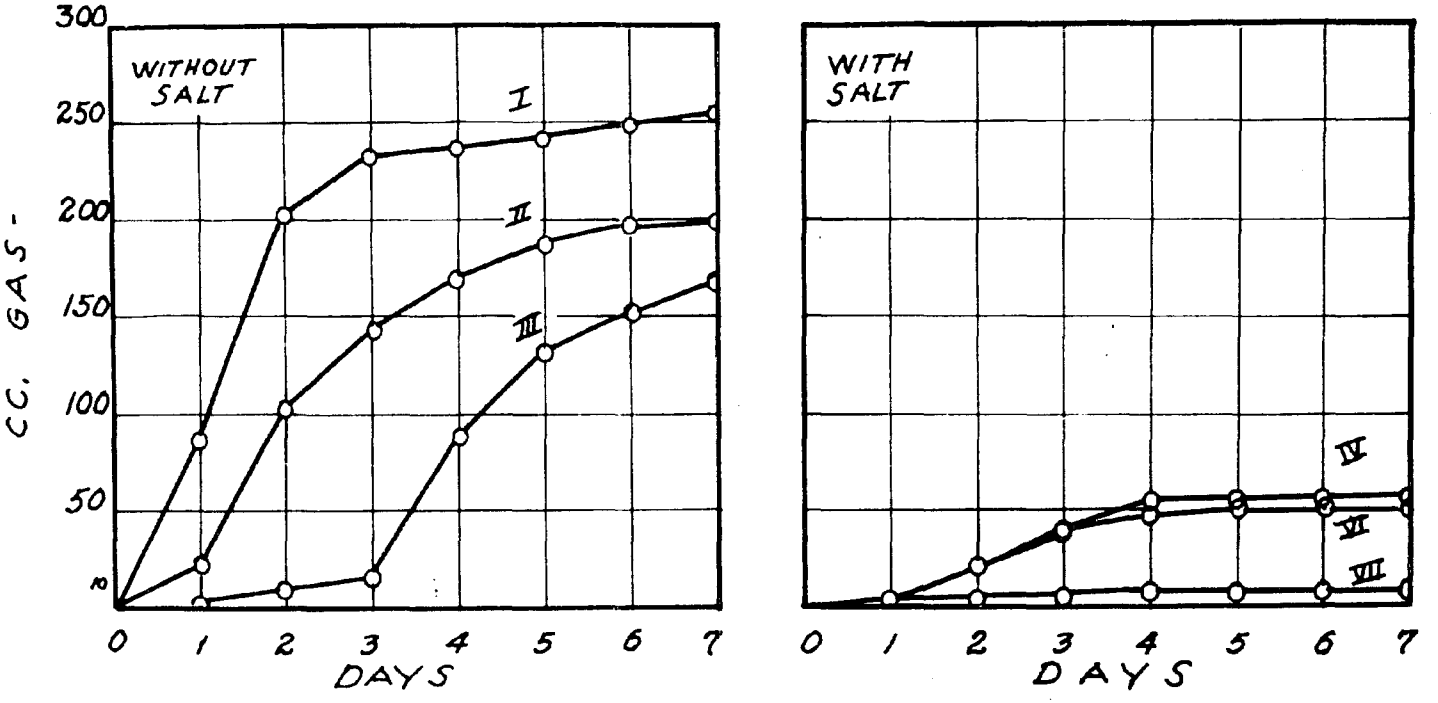

Fig. 14. Effect of autoclaving cucumber juice with and without salt (15 per cent saturation) on gas evolution. I, unheated; II, 40 min. heating; III, 100 min. heating; IV, unheated plus salt; VI, 40 min. heating plus salt; VII, 100 min. plus salt.

five-fold increase over the 100 minute heat treatment (III).

While the fermentation of the juice receiving the 100 minute heat treatment (III) evolved within 30 cc. of the amount of gas as compared to the 40 minute heat treatment (II), the latter was markedly retarded in gas evolution since only about one-tenth the volume of gas was produced in the three day interval.

In the series to which salt was added, two of the treatments shown, the unheated (IV) and the 40 minute heated (VI), were comparable as to the amount and rate of gas evolution. Here the major portion of the gas was evolved during the first three days. It is noted that heating the juice for 100 minutes (VII) resulted in very little gas production from the subsequent fermentation.

A summary of the findings of this study reveal the following: (a) Certain changes occur during the heating of cucumber juice which influenced the speed of fermentation as well as the amount of gas evolved; (b) the above influence resulted in a much delayed fermentation in juice receiving 100 min. heating as compared to a 40 minute heating although the final volumes of gas evolved after one week were comparable; (c) under the conditions of experimentation, it was not possible to bring about a more vigorous fermentation in the presence of salt (15 per cent sat.) in unheated juice since the fermentations resulting from unheated and 40 min. heated juice were similar with respect to speed of fermentation and the amount of gas evolved; (d) no significant influence could be demonstrated by the type of salt employed (plant salt or C.P. salt).

itially (after sterilization) and after the gas analyses had been completed.

Attention is called to the fact that with four compounds, lactose, raffinose, rhamnose and salacin, the one per cent solutions did not yield sufficient gas volumes for analyses which were comparable to the volumes resulting from the other carbon compounds. Hence, in these cases, the fermentations were repeated, tripling the original amount for each compound so that in the final results the figures are shown calculated on the basis of the fermentation of 50 cc. of a 3.0 per cent solution (1.5 g. of compound present). Also, in these four instances, the residual gas was incorporated into the collected gas at the time of analysis. However, the latter change of procedure is of no particular consequence since the final hydrogen to carbon dioxide ratios for all compounds fermented were calculated from the total volumes of the gases produced, both evolved and residual.

The results of the fermentations with respect to gas evolution and composition are presented in Table 36. A relative evaluation* as to the capability of type strain H-1438 to ferment the different substances tested, as evidenced by the amount of gas evolved (excluding residual gas which was similar in amount for all cases where the compounds were utilized to any extent) would be as follows:- Those readily fermented, dextrose, d-mannitol, d-galactose, d-mannose, saccharose and levulose; those less readily fermented, d-sorbitol and l-arabinose; those moderately fermented, maltose and xylose; those poorly fermented,

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* For the conditions under which this study was conducted and based on the evolved gas from the fermentation of 50 cc. of the various solutions.

lactose, raffinose, rhamnose and salacin.

In a study of the data concerning the composition of the gas evolved from these fermentations, it was found that the compounds fell into three general classifications with reference to production of hydrogen and carbon dioxide. In the first, the gas is composed of one volume of hydrogen and two volumes of carbon dioxide (1:2) and the compounds included are: l-arabinose, dextrose, d-galactose, levulose, d-mannose and saccharose (1 pentose, 4 hexoses and 1 disaccharide).

In the second, the gas is composed of one volume of hydrogen and one volume of carbon dioxide (1:1) and the compounds included are: l-xylose, rhamnose, maltose, raffinose, d-mannitol, d-sorbitol and salacin (2 pentoses, 1 disaccharide, 1 trisaccharide, 2 hexahydric alcohols and 1 glucoside).

In the third, the gas is composed of two volumes of hydrogen and one volume of carbon dioxide (2:1) and only one compound is included; lactose (disaccharide).

The volumes of residual gas and the analyses as to components from the above fermentations (Table 36) are shown in Table 37. The results show that carbon dioxide represents the major portion of the gas.

The rate of gas evolution for the fermentations presented in Table 38 shows, in general, that the major portion of the gas is evolved within 24 to 48 hours. In most cases, little or no gas evolution was noted after four days of incubation. The data from this table are shown graphically in Figure 15. Attention is called to the fact that the curves shown represent the gas evolved from the fermentation of 50 cc. of the test carbon compound solutions (1.0 per cent).

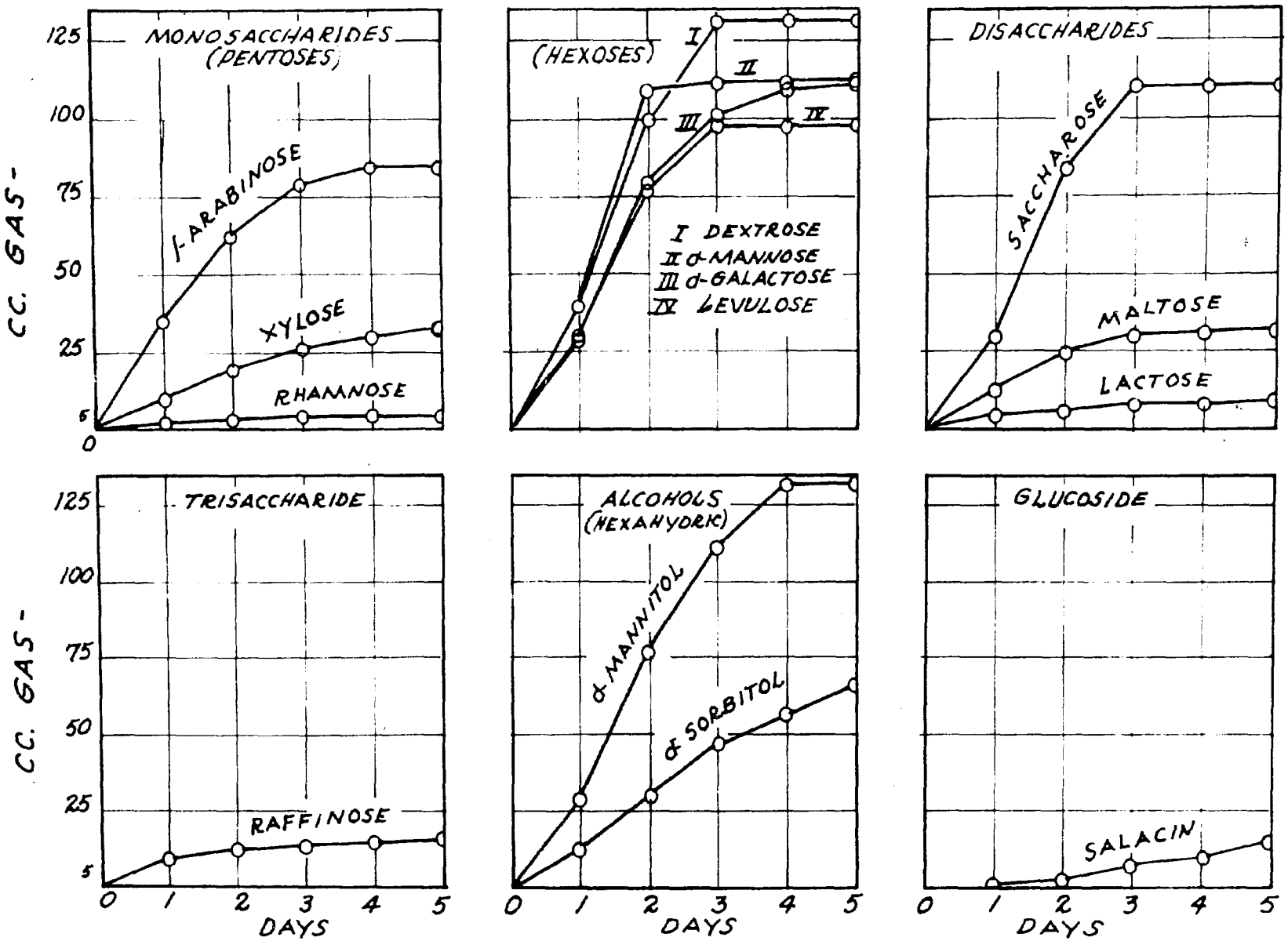

Fig. 15. Gas evolution from the fermentation of 50 cc. of one per cent solns. of various carbon compounds.

i. Effect of heat on viability of broth cultures.

Several experiments were conducted to determine the thermal death times for strains H-1438 and H-138. Twenty-four hour cultures grown in broth containing dextrose (0.5 per cent), tryptone (0.5 per cent), and K_2HPO_4 (0.5 per cent) were used. In the preliminary experiments, five cc. amounts of the cultures were placed in a water bath at the desired temperature. The water was circulated by a motor driven stirrer. At the same time the cultures were placed in the bath, a like tube of broth with an inserted thermometer was immersed, which served as a temperature control for the cultures. At the various temperature and time intervals, standard loops of the broth cultures were removed and transferred to sterile tubes of broth and incubated 48 to 72 hours at 35° C. and observed for growth.

In the first experiment the water bath was kept at 60° C. and loops of the broth cultures were removed when the latter had reached 40°, 50° and 60° C. as well as intervals of two, four, six, eight and 10 minutes at 60° C. The times for the broth tubes to reach 40°, 50° and 60° C. were one-half minute, one minute and two and one-half minutes respectively.

The results showed that strain H-138 survived 50° but was killed by exposure to 60° C. while strain H-1438 survived two minutes at 60° C. but was killed by an exposure for four minutes at the same temperature.

In the next experiment, the water bath was maintained at 50° C. and exposure intervals used were two, four, six, eight and 10 minutes. This temperature was selected in order to determine its effect, particularly on strain H-138 for longer exposure intervals.

In the above tests H-138 survived all exposure intervals. Strain H-1438 showed the same results, which was to be expected since it survived 60° C. for two minutes.

In the final experiment, the total number of organisms of strain H-1438 surviving temperatures of 40°, 50°, 55°, 60° and 60° C. for two, four and six minutes was determined. In this case, eight cc. of a 24 hour broth culture was used and at the above temperatures one cc. was removed and plated on ordinary destrose agar. The results obtained are tabulated below:

Temperature	Survival Count
° C.	per cc.
Initial* (30°)	1,920,000,000
40°	1,920,000,000
50°	1,008,000,000
55°	793,000,000
60°	23,000
60°(for 2 min.)	5,000
60°(for 4 ")	10
60°(for 6 ")	0

* At start of the experiment.

The above plate counts per cc., plotted logarithmically, are shown in Figure 16. Here, the very sharp downward trend of the survival curve indicates that the critical death temperature (60°) is being approached. Points A and B represent the organisms present after exposure for two and four minutes respectively at 60° C.

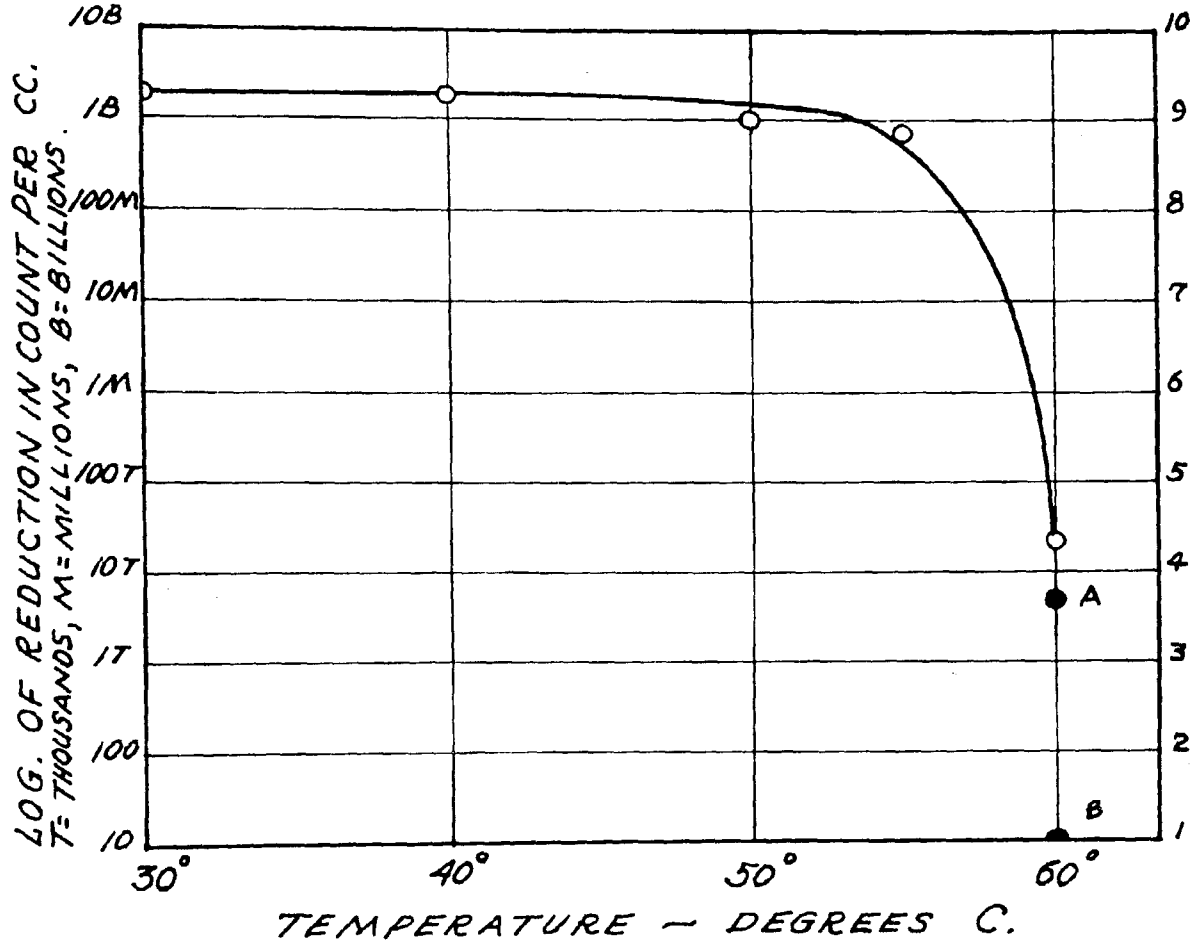

Fig. 16. Effect of temperature on the death rate of strain H-1438; A, two min. exposure at 60° C.; B, four min. exposure at 60° C.

Table 23. Fermentation of Dextrose Broth, Buffered at pH 5.15, by Several Strains of the Stock Culture Collection.

Strain	Outfit No.	Total vol. of gas*	Carbon dioxide		Hydrogen		Ratio of H_2:CO_2**	O_2	Remainder gas†	Residual gas	Gas accounted for by analysis
		cc.	%	cc.	%	cc.		cc.	cc.	cc.	%
H-739	1	139.3	69.0	96.1	27.9	38.9	1:2.47	.3	4.0	13.3	97.9
H-439	2	129.0	68.8	88.8	29.6	38.2	1:2.32	.3	1.7	12.3	99.4
H-639	3	145.7	70.0	102.0	28.0	40.8	1:2.50	.6	2.3	15.6	100.0
H-638	4	102.5	70.3	72.1	27.4	28.1	1:2.56	.3	2.0	12.3	99.2
H-1338	5	146.7	71.0	104.2	27.3	40.1	1:2.59	.3	2.1	17.0	99.3
H-438	6	140.0	70.8	99.1	27.3	38.2	1:2.59	.3	2.4	13.8	99.1
H-138	7	33.3	55.7	18.5	38.5	12.8	1:1.44	.3	1.7	4.4	99.2

* Values based on fermentation of 50 cc. of one per cent dextrose broth; gas volumes include residual gas.

** Calculated from volumes of H_2 and CO_2.

† Principally nitrogen, also includes experimental error.

Table 24. Rate of Gas Production During Fermentation of Dextrose Broth, Buffered at pH 5.15, by Several Strains from Stock Culture Collection.

Strain	Outfit No.	Total volume of gas collected in cc.*						
		1 day	2 days	3 days	4 days	5 days	6 days	7 days
H-739	1	49	107	113	116	116	116	120
H-439	2	30	98	105	109	109	109	109
H-639	3	75	125	125	125	125	128	130
H-638	4	39	75	78	80	81	82	83
H-1338	5	52	118	118	121	121	121	128
H-438	6	40	115	119	122	123	123	125
H-138	7	6	24	26	27	27	27	28

* Maximum amount of gas collected; does not include residual gas.

Table 25. Comparison of Four Fermentations of Dextrose Broth, Buffered at pH 5.15, by Strain H-1438

A. Gas composition

H-1438	Total volume of gas*	Carbon Dioxide		Hydrogen		Ratio** of H_2:CO_2	O_2	Remainder gas†	Residual gas	Gas accounted for by analysis
	cc.	%	cc.	%	cc.		cc.	cc.	cc.	%
1	132.7	68.6	92.4	29.3	37.6	1:2.45	.5	2.2	11.4	99.9
2	149.5	70.0	104.7	28.5	42.6	1:2.45	.3	1.9	17.0	99.5
3	148.6	70.2	104.3	28.1	41.8	1:2.49	.3	2.3	17.2	99.3
4	143.0	69.0	98.7	29.4	42.0	1:2.35	.3	1.8	15.0	99.9

B. Rate of Gas Production

H-1438	Total volume of gas collected in cc.††						
	1 day	2 days	3 days	4 days	5 days	6 days	7 days
1	34	113	115	116	117	120	120
2	65	130	130	130	130	130	131
3	44	120	122	124	126	128	128
4	52	124	126	128	128	128	128

* Values based on fermentation of 50 cc. of one per cent dextrose broth; gas volumes include residual gas.

** Calculated from volumes of H_2 and CO_2.

† Principally N_2, also includes experimental error.

†† Maximum amount of gas collected; does not include residual gas.

Table 26. Effect of Temperature Upon the Production and Composition of the Gas from Dextrose Broth Buffered at pH 5.15.

Temperature in C.	Volume of collected gas*	Carbon dioxide		Hydrogen		Ratio of H_2:CO_2	O_2	Remainder gas**	Residual gas	Gas accounted for by analysis
	cc.	%	cc.	%	cc.		cc.	cc.	cc.	%
5°	0								0	
13°	60.0	67.6	40.6	28.8	17.3	1:2.34	.2	1.9	29.5	98.1
19°	106.9	65.0	69.5	33.5	35.8	1:1.94	.2	1.4	11.3	99.5
24°	104.0	65.4	68.0	33.4	34.7	1:1.97	.2	1.1	12.1	99.8
30°	126.6	66.2	83.8	31.9	40.4	1:2.07	.3	1.1	18.4	99.1
35°	127.0	69.2	87.9	28.1	35.7	1:2.46	.5	2.9	13.0	99.3
40°	94.6	64.6	61.1	32.4	30.7	1:1.99	.4	2.4	12.4	99.0
45°	0								0	

* Values based on fermentation of 50 cc. of one per cent dextrose broth; gas volumes include residual gas.

** Principally N_2, also includes exp't. error.

Table 27. Effect of Temperature on the Rate of Gas Production in Dextrose Broth, Buffered at pH 5.15.

Temperature in C.	Total volume of gas collected in cc.							
	1 day	2 days	3 days	4 days	5 days	6 days	7 days	8 days
13°	0*	0*	3	5	10	16	22	25
19°	0*	7	25	41	62	71	87	87
24°	10	41	63	80	83	87	92	92
30°	26	72	88	93	98	102	105	105
35°	54	96	101	108	114	114	114	114
40°	33	45	50	57	65	74	78	82

* No gas collected, but residual gas present in the medium.

Table 28. Effect of pH Upon the Production and Composition of the Gas from Buffered Dextrose Broth.

pH	Volume of collected gas	Carbon dioxide		Hydrogen		Ratio of H_2:CO_2	O_2	Remainder gas**	Gas accounted for by analyses
	cc.	%	cc.	%	cc.		cc.	cc.	%
3.6	0								
4.25	54.0	54.0	36.8	38.5	21.5	1:1.71	.4	2.3	96.5
5.3	107.0	65.4	78.4	33.0	36.3	1:2.15	.3	1.4	99.9
6.0	87.4	65.4	68.3	32.4	29.8	1:2.29	.4	1.4	99.9
7.0	58.3	58.6	46.4	35.6	22.4	1:2.07	.4	2.9	97.4
7.55	59.8	56.4	44.6	39.7	25.2	1:1.76	.2	2.2	98.1
8.05	31.1	48.2	26.8	42.4	16.2	1:1.65	.4	1.8	96.6
8.85[i]	21.6	48.2	8.7	29.2	5.3	1:1.64	.4	4.2	88.4
6.8[c]	116.9	64.8	91.9	32.6	38.8	1:2.36	.4	2.7	98.9

* Figures represent total volumes of CO_2 and H_2 produced by the fermentation, including collected and residual gas.

** Principally nitrogen from air present above culture medium at start, also includes manipulation and experimental error.

t Ratios calculated from total volumes of CO_2 and H_2 produced from 50 cc. of one per cent dextrose broth.

i Residual gas included in original analyses of collected gas.

c Unbuffered control.

Table 29. Composition of Residual Gas from the Fermentations Tabulated in Table 28.

pH	Volume of residual gas*	Carbon dioxide		Hydrogen		Remainder gas **
	cc.	%	cc.	%	cc.	cc.
3.6	0					
4.25	9.8	77.0	7.6	7.4	.7	1.5
5.3	10.7	78.6	8.4	9.3	1.0	1.3
6.0	14.0	79.5	11.1	9.7	1.4	1.5
7.0	15.2	80.0	12.2	10.2	1.6	1.4
7.55	13.7	79.5	10.9	11.2	1.5	1.3
8.05	16.6	79.0	11.8	15.5	2.3	0.8
8.85[i]						
6.8[c]	18.7	86.5	16.2	3.7	.7	1.9

* Gas dissolved in culture medium and present above culture medium; all calculations based on 50 cc. of one per cent dextrose broth fermented.

** Principally air introduced during analysis manipulation, also includes experimental error.

i Residual gas analysed with the collected gas.

c Unbuffered control.

Table 30. Effect of pH Upon the Rate of Gas Production in the Fermentation of Dextrose in Broth.

pH	Total volume of gas collected in cc.*					
	1 day	2 days	3 days	4 days	5 days	6 days
4.25	10	44	61	61	61	61
5.3	53	101	110.	110	110	110
6.0	49	76	85	89	89	89
7.0	39	45	49	53	60	60
7.55	39	46	53	56	60	60
8.05	16	23	26	29	32	32
8.85	2	3	5	7	9	9
6.8[c]	34	83	112	118	118	118

* Figures based on 50 cc. of dextrose broth fermented.
c Unbuffered control.

Table 31. Effect of Salt (NaCL) Concentration Upon Gas Production and Composition During the Fermentation of Dextrose Broth and Cucumber Juice Broth.

Salt concent.		Vol. of collected gas*	Carbon dioxide		Hydrogen		Ratio of H_2:CO_2	O_2	R. gas	Rs. gas	pH**	Gas acc't. for by analyses
% sat.	g/100 cc.											
		cc.	%	cc.	%	cc.		cc.	cc.	cc.		%
A. Dextrose broth buffered at pH 5.15												
0	0	126.9	66.8	84.8	31.1	39.5	1:2.14	.3	2.3	13.1	5.15	99.0
5	1.33	125.0	68.0	85.0	28.7	35.9	1:2.36	.5	3.6	13.0	4.9	98.7
10	2.68	74.2	63.8	47.3	32.3	23.9	1:1.97	.5	2.5	9.6	4.75	99.0
15	4.06	13.1	48.0	5.4	45.7	5.2	1:1.03	--	2.5	6.1	4.65	93.7
20	5.47	0	--	--	--	--	--	--	--	0	4.6	--
25	6.91	0	--	--	--	--	--	--	--	0	4.55	--
B. Cucumber juice broth unbuffered												
0	0	(lost)									5.9	
5	1.33	208.6	79.4	165.6	19.25	40.3	1:4.10	.4	2.3	16.7	5.7	99.6
10	2.68	101.5	80.8	82.0	16.0	16.2	1:5.06	.4	2.9	14.0	5.65	98.2
15	4.06	63.6	79.2	49.3	15.6	8.9	1:5.53	.4	5.0	8.5	5.6	97.8
20	5.47	29.0	74.6	21.6	14.5	4.2	1:5.14	.5	2.5	12.6	5.55	98.1
25	6.91	0	--	--	--	--	--	--	--	0	5.5	--

* Includes residual gas; calculations based on 50 cc. of one per cent dextrose broth, and 50 cc. of two per cent cucumber juice broth.

** pH values taken at start of fermentation, drop in pH due to influence of NaCl.

R. Remainder gas.

Rs. Residual gas.

Table 32. Effect of Salt (NaCl) Concentration Upon the Rate of Gas Production in the Fermentation of Dextrose Broth and Cucumber Juice Broth.

Salt concentration		Total volume of gas collected in cc.						
% sat.	g./100cc.	1 day	2 days	3 days	4 days	5 days	6 days	7 days
Dextrose broth*								
0	0	72	103		113	114	114	114
5	1.33	72	105		108	108	112	112
10	2.68	21	37	46	58	64	64	64
15	4.06	0[d]	0[d]	2	4	5	6	7
20	5.47	0	0	0	0	0	0	0
25	6.91	0	0	0	0	0	0	0
Cucumber Juice broth**								
0	0	(lost)						
5		50	133	180	189	192	195	195
10		16	74	79	84	88	92	93
15		2	29	33	41	47	52	55
20		0[d]	1	3	11	15	16	16
25		0	0	0	0	0	0	0

* Figures based on 50 cc. of one per cent dextrose broth.
** Figures based on 50 cc. of two per cent cucumber juice broth.
d No collected gas, but gas present in the medium.

Table 33. Effect of Heat and NaCl Upon Gas Production and Composition in the Fermentation of Fresh Cucumber Juice.

Treatment of Seitz filtered juice	Volume of collected gas	Carbon dioxide		Hydrogen		Ratio of $H_2:CO_2$	O_2	R.	Rs.	Initial pH	Final pH
	cc.	%	cc.	%	cc.		cc.	cc.	cc.		
I Unheated	275.6	77.0	212.2	22.0	60.6	1:3.50	.6	2.2	19.4	6.3	5.95
II + 40 min. autoclaving**	207.6	78.6	163.2	19.8	41.1	1:3.97	.6	2.9	17.7	6.1	5.55
III + 100 min. autoclaving.	185.2	78.6	145.6	19.2	35.6	1:4.08	.4	3.6	14.5	5.65	5.5
IV Unheated + 15% sat. C.P. NaCl	69.0	81.7	56.4	11.6	8.0	1:7.05	.4	4.2	13.5	6.2	5.4
V Same as IV with plant salt	53.7	83.2	44.7	8.3	4.5	1:9.33	.4	4.1	9.9	6.2	5.4
VI + 15% sat. C.P. NaCl + 40 min. autoclaving	64.1	83.5	53.5	10.4	6.7	1:7.98	.3	3.6	14.1	5.85	5.3
VII Same as VI + 100 min. autoclaving	14.5	79.0	11.5	15.6	2.3	1:5.00	.2	.5	6.0	5.35	5.18

* Includes residual gas; calculations based upon 50 cc. of juice fermented.
** Autoclaved at 15 lbs. pressure.
R. Remainder gas.
Rs. Residual gas.

Table 34. Effect of Heat and NaCl Upon the Rate of Gas Production in Fresh Cucumber Juice.

Treatment of Seitz filtered juice	Total volume of gas collected in cc.*						
	1 day	2 days	3 days	4 days	5 days	6 days	7 days
I Unheated	88	202	234	238	242	249	256
II + 40 min. autoclaving	20	101	144	169	187	196	196
III + 100 min. autoclaving	3	8	13	88	131	151	168
IV Unheated + 15% sat. C.P. NaCl	1	21	40	56	56	56	56
VI + 15% sat. C.P. NaCl + 40 min. autoclaving	1	21	39	48	50	50	50
VII Same as VI + 100 min. autoclaving	0[d]	3	5	8	8	8	8

* Figures based on 50 cc. of juice fermented.

d No collected gas, but residual gas present in the medium.

Table 35. Effect of Dextrose (0.1 g.) Upon Maltose Fermentation.

A. Total gas production from dextrose and maltose mixture.

Calculated amount of gas resulting from 0.1 g. of dextrose based on dextrose fermentation...	28.0 cc.
Calculated amount of gas resulting from 0.4 g. of maltose based on maltose fermentation.....	30.5
Total	58.5 cc.
Total* amount of gas produced from the mixture of 0.1 g. of dextrose and 0.4 g. of maltose.....	54.4 cc.
Difference between fermentation....	4.1 cc.

B. Rate of Gas Production in the Mixture of Dextrose and Maltose Compared to Maltose Alone.

Total volume of collected gas in cc.

	1 day	2 days	3 days	4 days	5 days	6 days
0.1 g. of dextrose + 0.4 g. of maltose	13	19	24	28	32	36
0.5 g. of maltose alone	12	24	30	31	31	31

* Includes residual gas.

Table 36. Production and Composition of Gas from Carbon Compounds in 0.5 Tryptone Broth by Strain H-1438.

Compound (0.5 g.)	Vol. of collected gas	Carbon dioxide		Hydrogen		Ratio of H_2:CO_2	O_2	Remainder gas	Gas accounted for by analysis
	cc.	%	cc.*	%	cc.*		cc.	cc.**	%
l-Arabinose	83.1	59.1	57.4	34.1	28.7	1:2.00	.3	5.4	95.3
Dextrose	131.3	66.2	93.3	31.8	42.7	1:2.18	.3	2.2	99.0
d-Galactose	111.5	60.8	73.2	36.8	41.5	1:1.76	.5	2.2	99.6
Lactose[tt]	46.6	30.6	14.2	64.2	30.4	2.14:1	.3	1.7	97.8
Levulose	98.3	63.0	69.0	31.5	32.1	1:2.14	.4	4.9	96.5
Maltose	30.5	36.6	15.9	53.3	17.4	1.09:1	.2	2.8	92.9
d-Mannose	110.8	66.6	79.4	28.1	33.7	1:2.50	.4	5.5	96.7
d-Mannitol	131.8	48.6	69.3	50.0	67.1	1:1.03	.3	1.4	99.6
Raffinose[tt]	58.7	41.3	24.2	53.7	31.5	1.30:1	.5	2.8	97.2
Rhamnose[tt]	52.8	44.6	23.6	49.5	26.1	1.10:1	.3	2.9	96.6
Saccharose	109.7	63.8	75.7	29.4	33.5	1:2.25	.7	6.7	96.2
Salacin[tt]	58.6	42.7	25.0	52.4	30.7	1.22:1	.2	2.7	97.1
d-Sorbitol	85.9	40.0	41.3	56.8	50.7	1.22:1	.4	2.3	99.3
l-Xylose	34.6	32.3	18.0	56.0	20.7	1.15:1	.4	3.6	94.9

* Figures represent total volumes of CO_2 and H_2 produced from the fermentations and include collected gas as well as residual gas.

** Principally nitrogen from the air present above the culture medium at start of fermentation, also includes experimental error.

t Ratios calculated from the total volumes of H_2 and CO_2 produced from 50 cc. of one per cent solutions.

tt The original gas volumes from 50 cc. of one per cent solutions in order listed above were 9.0, 16.8, 9.1 and 13.1 cc. respectively; since these volumes were too small for accurate analyses the fermentations were repeated. Figures in the above table are based on total gas volume from fermentation of 50 cc. quantities of three per cent solutions of each carbohydrate.

Table 37. Composition of Residual Gas From Fermentations Shown in Table 36.

Compound	Volume of residual gas*	Carbon dioxide		Hydrogen		Remainder gas	pH of soln.	
							at start of ferm.	at finish of ferm.
	cc.	%	cc.	%	cc.	cc.		
l-Arabinose	10.1	82.0	8.3	4.2	.4	1.4	6.65	4.6
Dextrose	8.8	72.0	6.3	10.5	.9	1.6	6.9	5.05
d-Galactose	8.9	61.0	5.4	5.8	.5	3.0	6.9	4.75
Lactose**	--						7.05	4.5
Levulose	9.8	71.0	7.0	11.5	1.1	1.7	6.95	4.55
Maltose	7.6	61.4	4.7	14.3	1.1	1.8	--	4.55
d-Mannose	7.9	71.0	5.6	7.3	.6	1.7	6.95	4.7
d-Mannitol	7.6	69.0	5.2	14.9	1.1	1.3	7.15	4.9
Raffinose**	--						7.15	4.3
Rhamnose[t]	--						7.0	4.7
Saccharose	8.5	67.4	5.7	13.5	1.2	1.6	7.05	4.65
Salacin**	--						7.05	4.35
d-Sorbitol	9.7	70.8	6.9	19.1	1.9	0.9	7.1	5.2
l-Xylose	9.7	70.0	6.8	13.7	1.3	1.6	6.75	4.7

* Gas dissolved in culture media and present above the media. All calculations based on 50 cc. of broth containing one per cent of the carbon compound.

** Not sufficient gas for analysis.

t Residual gas included at time of analyses of collected gas.

Table 38. Rate of Gas Production From Different Carbon Compounds by Strain H-1438.

Compound*	Total volume of gas collected in cc.					
	1 day	2 days	3 days	4 days	5 days	6 days
l-Arabinose	35	62	79	84	84	84
Dextrose	39	100	131	131	131	131
d-Galactose	30	79	101	109	112	113
Lactose	4	5	8	8	9	9
Levulose	28	77	98	98	98	98
Maltose	12	24	30	31	31	31
d-Mannose	39	109	111	111	111	111
d-Mannitol	29	77	112	132	132	132
Raffinose	9	12	14	15	16	17
Rhamnose	2	3	4	4	4	4
Saccharose	30	84	110	110	110	110
Salacin	0**	2	7	10	12	13
d-Sorbitol	12	30	48	57	63	72
l-Xylose	10	19	26	30	32	32

* Figures based on 50 cc. of broth containing one per cent of the carbon compound.

** No collected gas, but residual gas present in the medium.

FERMENTATIONS UNDER COMMERCIAL CONDITIONS

The experimental work presented up until this point has dealt with isolation, identification and biochemical studies of the organisms responsible for the evolution of hydrogen during cucumber fermentations.

The concluding portion of the experimental observations, deals principally with the typical fermentations of these organisms under commercial salting conditions. The fermentations were followed as to their progressive changes in gas evolution, gas composition and yeast populations.

The work reported is based on studies made during the 1940 season.

Experimental Procedure

In general the salting procedure was as follows: Vats of 85 bushel capacity (see Figure 17) were filled with cucumbers, fitted with false heads and salt brine added so as to come a few inches above the heads. Initial brine concentrations of 20, 40 and 60° salometer (per cent saturation with respect to salt) were used. The actual salting schedule, showing the rate of increase of brine concentration for each treatment is shown in Table 39. The salting procedures will be indicated throughout the work by the initial salt concentration.

Table 39. Salting Schedule (1940).

Initial brine concentration	Rate of increase of brine concentration	Number of vats followed
° salometer	° salometer	
20	up 10° per week to 60°	2
40	up 5° per week to 60°	3
60	held at 60°	2

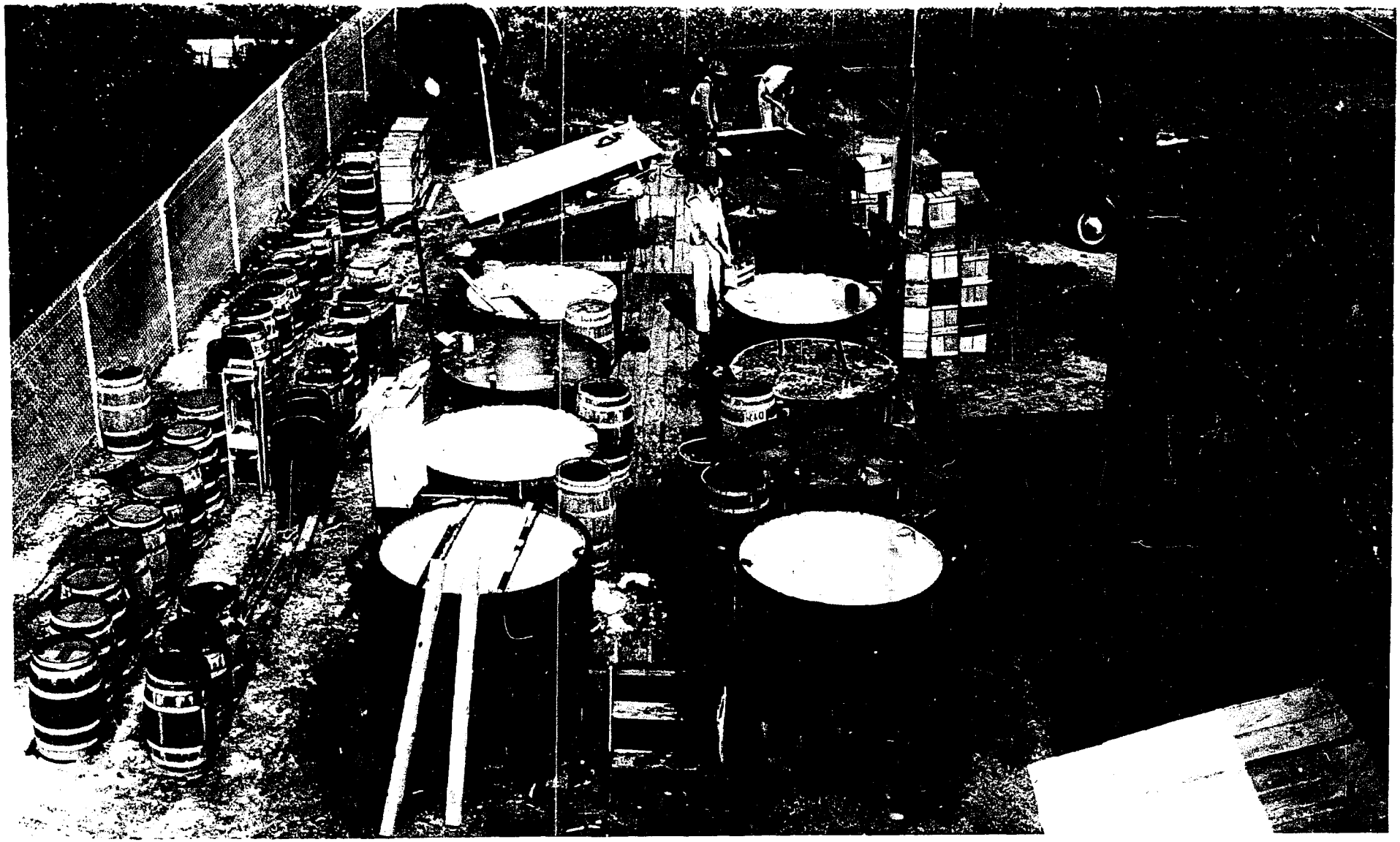

Fig. 17. Experimental vats (8½ bu. capacity) of the U. S. Dept. of Agric., located at the Chas. F. Cates Company, Faison, N. C. At the time the photograph was taken, most of the vats had been put down and fermentation was under way; two vats (5th and 7th in right row) are being prepared prior to filling with cucumbers.

In addition to the vats listed above, seven others were followed to the extent of single gas analyses during active fermentation. The latter vats were located at another plant and were salted similar to the 40° brine treatment, except that salt and water were added during the filling of the vats with cucumbers in amounts sufficient to make an initial 40° brine.

The prevailing brine temperature during active fermentation (two to six weeks) was within the range of 76 to 80° F. All vats were unsheltered.

The comparative gas evolution studies were based on the amount of gas collected from a representative portion of the surface area of each vat. The procedure was as follows: At the time each vat was salted, an inverted stainless steel funnel* 14 inches in diameter was placed just below the false head in the vats. It was held in place by running the delivery tube end of the funnel through a hole bored in the head. The trapped gases were collected over brine in glass bottles of three to five gallon capacity. At regular intervals the collected gases were removed by displacement into graduated one gallon containers and measured to one-tenth of one liter. Gas evolution determinations were carried out from the time the vats were put down until significant gas evolution had ceased (about one month).

For the major portion of the gas analyses (40° to 60° treatments), the samples were collected from the surface of the brine in the following manner: An inverted glass funnel supplied with a short piece of rub-

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* The ratio of the portion of the surface area occupied by the funnel (153.9 sq. in.) to the whole surface area of the vat (4,071.5 sq. in.) was 1:26.4.

ber tubing and a pinch clamp was placed over a crack in the false head and the evolved gases collected by displacement. When approximately 200 cc. of gas were collected, the sample was transferred by displacement into the receiving bottle of the sampling outfit shown on the top of the vat in Figure 18. By having a complete set of funnels and gas transfer bottles reserved for each vat under observation, samples could be taken quickly and at regular intervals and the gas analyzed without unnecessary delay. This was of considerable help in the cases where analysis was made three and four times a day during active gas evolution. The gas analyses for the two 20° fermentations (of which only one is reported) were obtained from the three gallon capacity collection bottles, used for the measurement of gas evolution. The procedure was essentially the same as that just described.

The method of analysis for the gas samples has been described in detail elsewhere in this report. The determination for oxygen was made on all samples but the values, being too small to be of any significance, are omitted from the results. During active evolution, these values always fell between 0.2 and 0.4 per cent. During slow evolution of gas from the fermentations, either at the start or during the lag between the hydrogen and yeast fermentations or at the conclusion of the fermentation proper, the values for oxygen increased slightly. This was due to the more or less constant rate of diffusion of air from the cucumber tissue throughout the curing period and hence oxygen would show up more clearly when there was slight evolution due to microbial activity. Analysis for methane was made on all gas samples and none was found.

Brine samples were taken for bacteriological analysis by inserting a piece of stainless steel tubing through an opening in the head of the

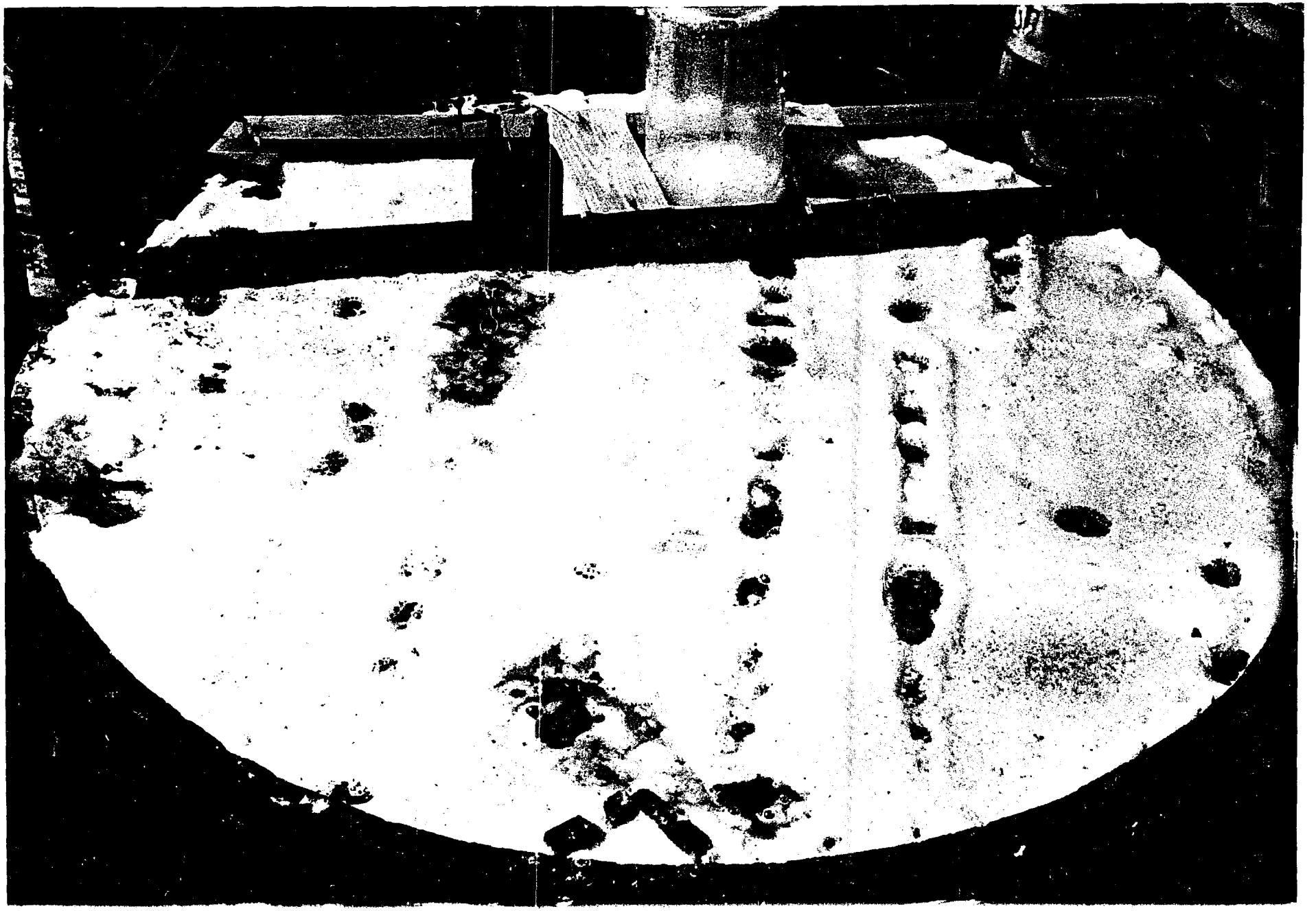

Fig. 18. Typical, vigorous hydrogen fermentation in a 40° salometer brine (Vat 4). Gas sampling transfer outfit (two 12 oz. bottles taped together) is shown at the left on the cross-board on the top of the vat. Just below, submerged in the brine is the inverted glass funnel used for collecting surface gas samples. The five gallon bottle (full of gas) set over the stainless steel funnel is shown in the background.

vat, down into the brine toward the center of the vat and withdrawing the brine sample through an attached piece of rubber tubing. Two 12 oz. bottles full were withdrawn before taking the final sample. Samplings in all cases were started from about the time the vats were put down and headed and were continued at regular intervals (one to two days) during the course of fermentation.

All fermentations were examined with respect to yeast populations in the brine. This was accomplished by plating dilutions of the brine on tartaric acid agar* (20). In brief this medium consisted of ordinary dextrose agar to which five cc. of sterile five per cent tartaric acid were added to 100 cc. amounts of the agar prior to pouring the plates. The addition of the tartaric acid brought the pH of the medium to approximately 3.7, thus inhibiting all the usual brine organisms except the yeasts. In the case of brine samples from the 60° fermentations, it was found necessary to decrease the amount of tartaric acid to three cc. per 100 cc. of medium since the yeasts from the above fermentations grew poorly when the greater amount of acid was used. The yeast plates were usually incubated three days at 35° C. and counted. In cases where growth was sparse, the incubation period was extended to five days.

Two 40° fermentations (Vats 4 and 6) were followed with respect to the populations of the organisms responsible for the evolution of hydrogen (the Aerobacter). An estimation of their numbers was based upon the colonial characteristics on nutritive caseinate agar (Difco),

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* Laboratory Manual (Methods of Analyses of Milk and Its Products) Int. Assn. Milk Dealers, p. 81 (1933).

containing eight cc. of 0.4 per cent brom-cresol-purple indicator added at the time of preparation of the agar.

The fermentations at 20, 40 and 60° salometer brine concentration will be discussed in the reverse order named. The results are shown in graphic form with respect to yeast populations, gas evolution and the composition of the evolved gas. This material is presented in two units, the upper one deals with yeast populations and gas evolution while the lower one shows the composition of the evolved gas with respect to carbon dioxide and hydrogen. The tabular data are included for specific reference (Tables 42, 43 and 44).

The values for yeast populations are plotted logarithmically, principally to facilitate showing counts prior and subsequent to active fermentation as well as counts that vary greatly during the active fermentation. Counts less than 1,000 per cc. are shown below the double line drawn parallel to the abscissa, opposite 1T on the ordinate. values less than 100 per cc. are not plotted.

The gas evolution values for all fermentations (20, 40 and 60° salometer treatments) are plotted according to the same scale (140 liters divided into seven 20 liter divisions). This permits comparison of the typical, vigorous hydrogen fermentations at 60° (Vats 3 and 9) and 40° (Vats 4 and 6) with those at 20 and 40° which resulted in little hydrogen evolution (Vats 8 and 10).

Discussion of Results

60° Salometer Brines

Over a period of several seasons, probably the 60° treatment shows the most consistent behavior with respect to what might be termed the

typical hydrogen fermentation and during this phase of the fermentation proper, the evolved gas consists of approximately equal portions of hydrogen and carbon dioxide. The data for duplicate fermentations at the above salt concentration are shown in Figure 19. The material is presented with respect to progressive changes in yeast populations, gas evolution (upper part) and composition of the gas evolved (lower part). Both fermentations are so strikingly similar that they can be discussed jointly.

The data indicate that the gaseous fermentation proper, starting on about the 11th day for both vats, was divided into two phases. The first, which covered a period of about one week, was brought about by the hydrogen-producing bacteria of the genus Aerobacter. Throughout this phase of active gas evolution, it will be noted (lower parts of Figure 19) that the percentage of hydrogen was relatively high (40 to 50 per cent). During the short interval (two to three days) of very slow gas evolution that followed the above fermentation, the gas that was collected for analyses came principally by diffusion from the interiors of the "bloated" or hollow cucumbers which were formed during the active hydrogen fermentation. This accounts for the presence of considerable amounts (about 30 per cent) of hydrogen during this interval.

The advent of the active yeast fermentation, on about the 16th to 18th day, brought about the second phase of active gas evolution. This is demonstrated by the upward trend of the gas evolution curve. The yeast fermentation covered a period of about 10 to 12 days which compares favorably to the period during which progressive gas evolution

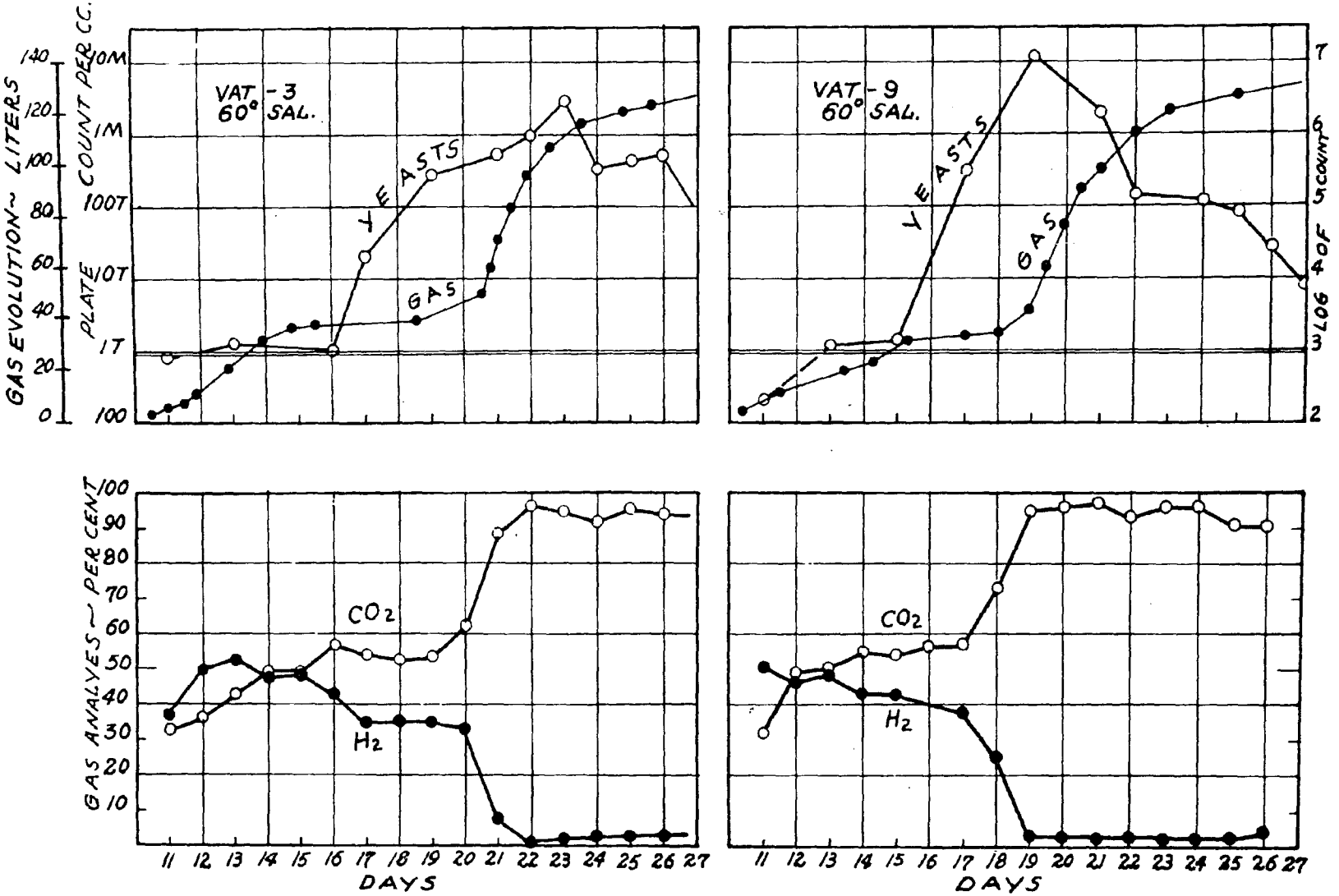

Fig. 19. Yeast populations, gas evolution and analyses of the gas from fermentations in 60° salometer brines from Vats 3 and 9.

was recorded. During the above fermentation, the gas consisted of practically all carbon dioxide although small amounts of hydrogen still persisted due to continued slow diffusion of gas from the bloaters.

Of the total amount of gas evolved from the fermentations, approximately one-fourth was produced by the organisms of the Aerobacter genus while the major portion was brought about by the yeasts.

40° Salometer Brines

In general, it has been observed that the typical, active hydrogen fermentation (see Figure 18) may or may not occur with the 40° salometer salting treatment. However, during the 1940 season a number of very active fermentations at this salt concentration were discovered. The data from duplicate treatments (Vats 4 and 6) are presented graphically in Figures 20 and 21. In addition to the curves shown for yeast populations, gas evolution and gas composition*, estimates of the numbers of Aerobacter present are also plotted (curves labelled A).

Generalized observations for both fermentations (Vats 4 and 6) show that the active hydrogen fermentation started within three to five days and lasted for a period of about four to six days. During this period, the gas evolution curves show a sharp upward trend which corresponds with the active growth phase of the Aerobacter group in the brine. The composition of the gas throughout this portion of the fermentation proper was similar with respect to proportions of hydrogen and carbon dioxide found present. Near the conclusion of active evolution (8th to 9th day) the proportion reached 1:1 (H_2 to CO_2).

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* For these two fermentations, the gas analyses values plotted represent the mean for three or four daily samples taken during the period of vigorous hydrogen evolution.

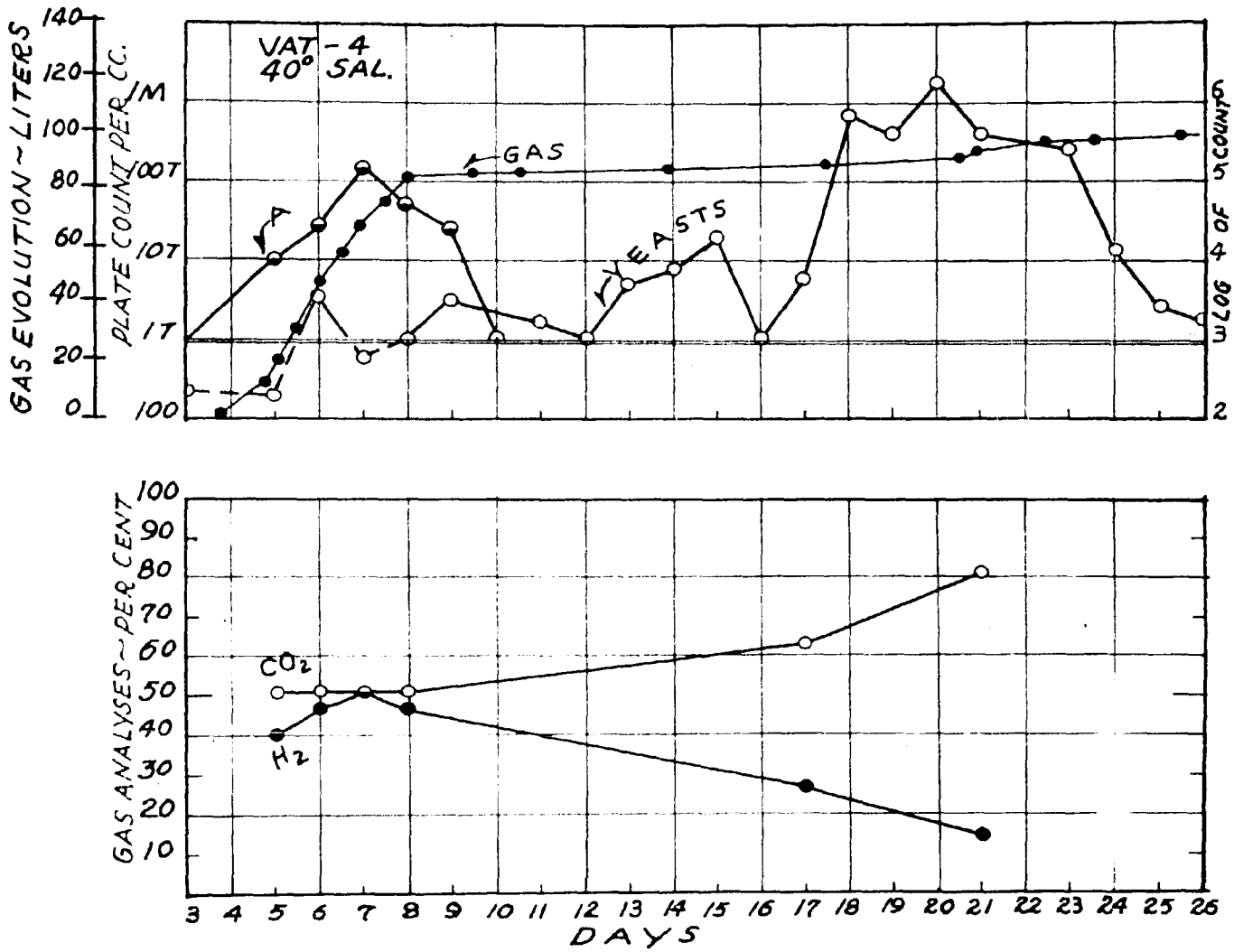

Fig. 20. Yeast populations, Aerobacter populations (curve labeled A), gas evolution and analyses of the gas from a fermentation in 40° salometer brine from Vat 4.

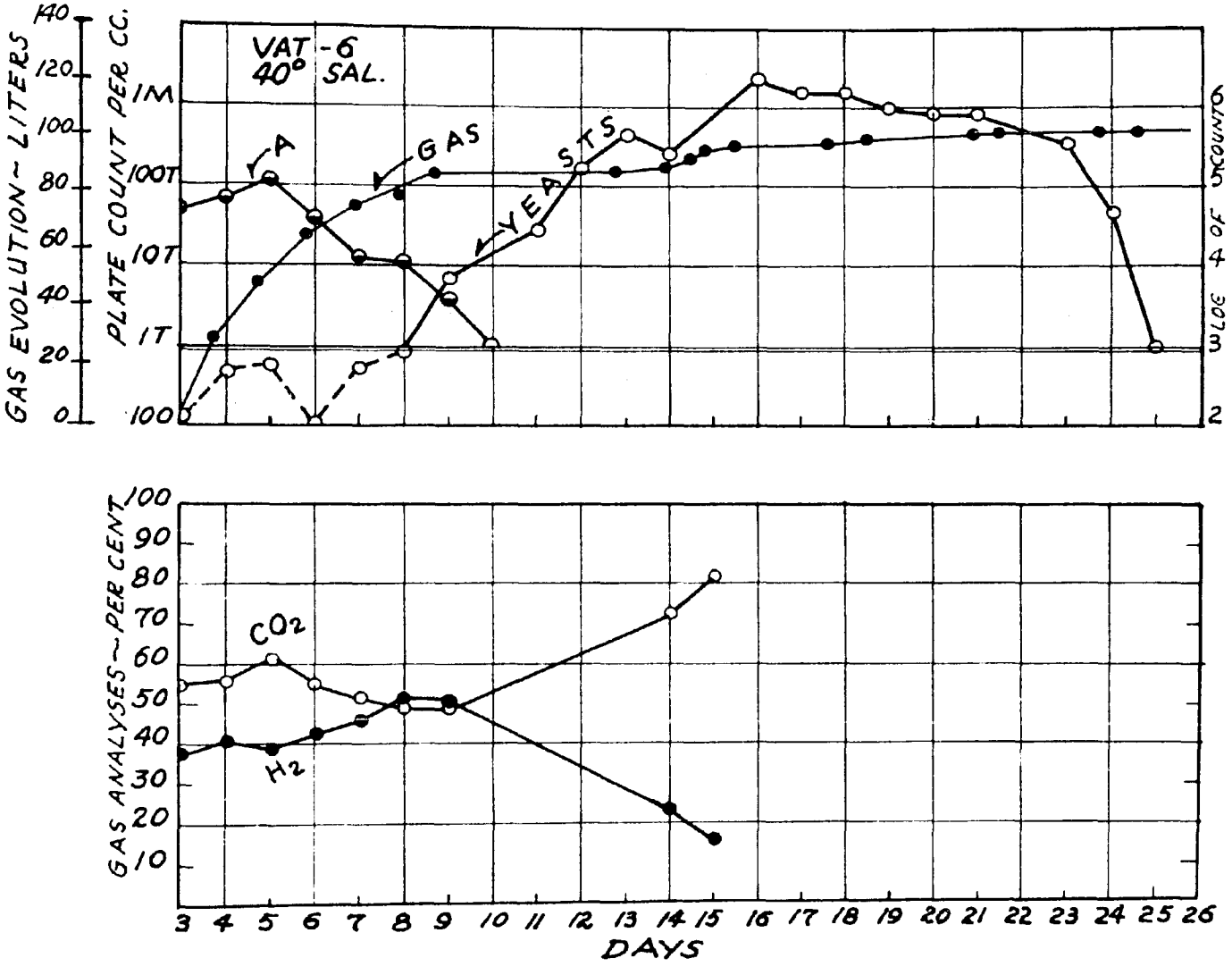

Fig. 21. Yeast populations, Aerobacter populations (curve labeled A), gas evolution and analyses of the gas from a fermentation in 40° salometer brine from Vat 6.

It is evident from comparison of the two fermentations in Vats four and six that some variation existed with regard to the onset of the respective yeast fermentations. In the case of the former (Vat 4) there was an interval of about nine days during which gas evolution was very slight, prior to the advent of the yeast fermentation. In the case of the latter fermentation (Vat 6) this interval was about five days duration. However, in both cases, the amount of gas produced by the yeast fermentation represented only a small portion of the total gas produced by the fermentation proper. The analyses of the few surface gas samples that could be collected during the above fermentation showed that carbon dioxide was the principle component. However, small amounts of hydrogen were also found. The presence of the latter was due to the slow diffusion of gas from the interiors of the bloated cucumbers formed during the previous hydrogen fermentation. This was the same relationship previously shown for the 60° brines.

Of the total amount of gas evolved from the 40° fermentations, approximately four-fifths was produced by the organisms of the Aerobacter group while the remainder was produced by the yeasts. This is an interesting observation since in the 60° brines almost the reverse was true while in both cases (40° and 60° brines) the significant yeast counts were similar in numbers and they occurred over comparable periods of time.

In Figure 22, gas evolution curves for single, active hydrogen fermentations at 40 and 60° (Vats 6, 9, previously discussed) are shown in such a manner as to emphasize the amount of gas produced by each phase of active fermentation as well as the duration of the evolution. This

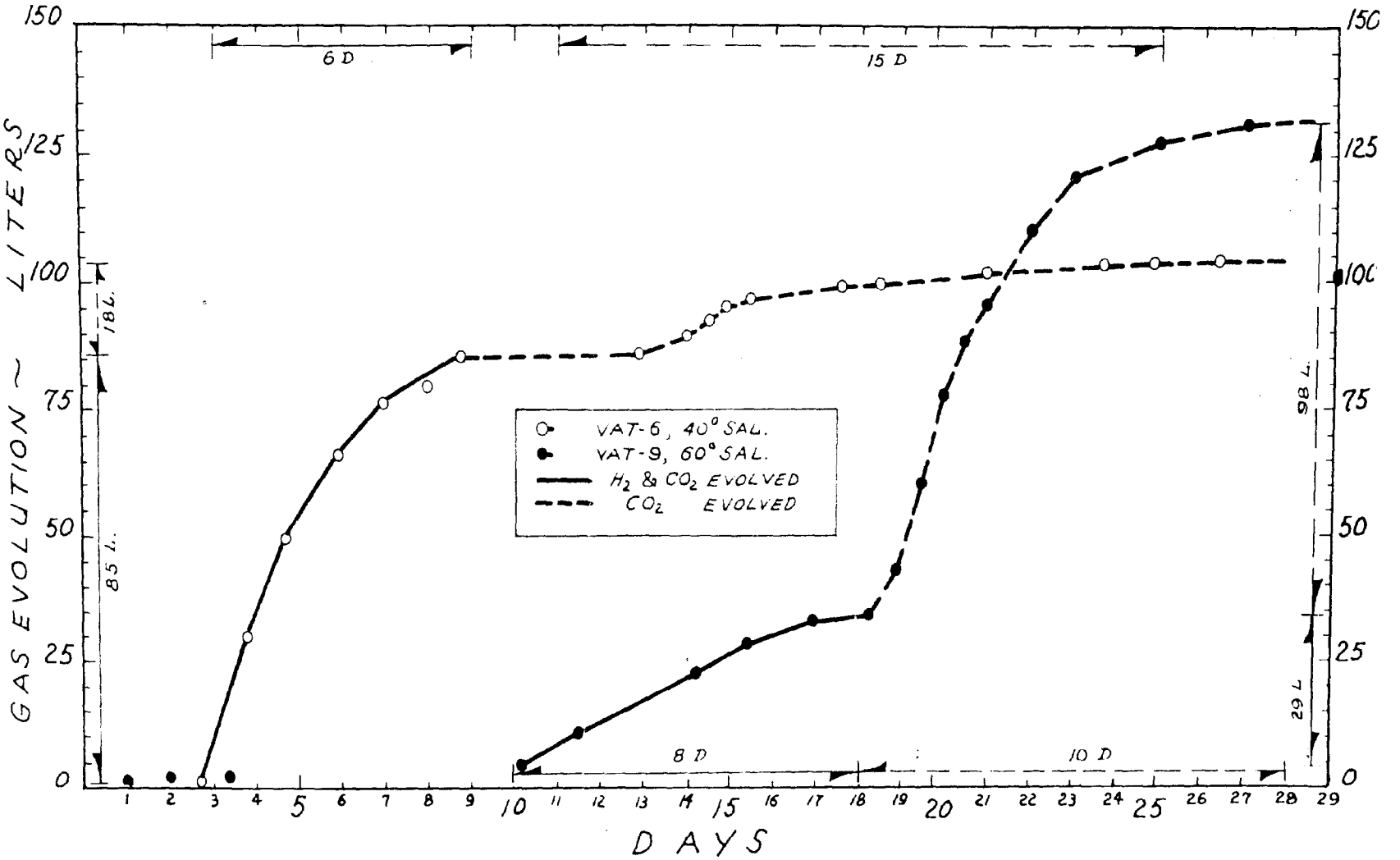

Fig. 22. Comparison of gas evolution from fermentations in 40 and 60° salometer brines. L, liters of gas collected from the 14 in. diam. funnels; D, time in days for each phase of evolution showing the H_2 and CO_2 curve for the hydrogen fermentation and the CO_2 curve for the yeast fermentation.

graph shows more clearly what has already been discussed with regard to gas evolution for these two typical hydrogen fermentations. Based on the amount of gas trapped by the 14 inch funnel (approximately one-twenty-fifth of the total fermentation), it is noted that for the 40° fermentation, which started on about the third day, approximately 100 liters of gas were collected of which the major portion, about 85 liters, was produced by the hydrogen fermentation (H_2 and CO_2 curve). This amount was produced in about the first six days after active evolution was recorded. The balance of the collected gas, about 18 liters, came from the subsequent yeast fermentation (CO_2 curve) over a period of about two weeks time (10th to 25th day).

The 60° fermentation, which started on about the tenth day, produced gas to the extent that approximately 130 liters were collected from the 14 inch funnel. Of this amount, about 29 liters resulted from the hydrogen fermentation (H_2 and CO_2 curve) during an interval of approximately eight days (10th to 18th day) while the major portion, 98 liters, was produced by the yeast fermentation (CO_2 curve) during the subsequent 10 day period (18th to 28th day).

Thus far, the material presented has dealt entirely with the typical, active hydrogen fermentations in 60 and 40° brines. However, it has been mentioned that the latter treatment (40°) may or may not result in the production of considerable amounts of hydrogen. Figure 23 shows the behavior of a 40° fermentation which resulted in only a small amount of hydrogen evolved. Here it is noted that the active yeast fermentation started on about the eleventh day and continued until about the twenty-second day. During this period the gas evolution curve shows a progressive increase. The gas analyses show that during the active

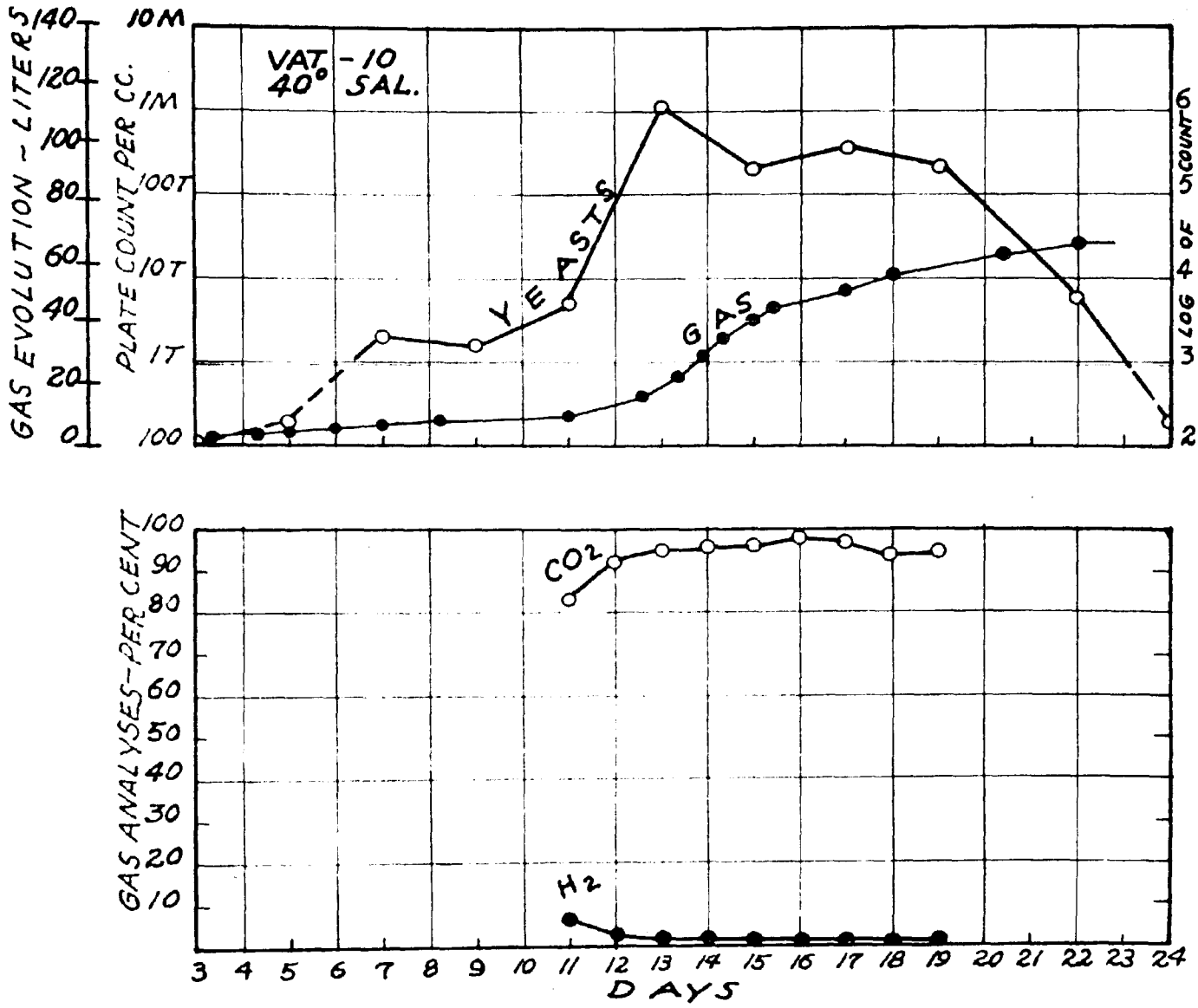

Fig. 23. Yeast populations, gas evolution and analyses of the gas from a fermentation in 40° salometer brine from Vat 10.

fermentation the evolved gas consisted principally of carbon dioxide, usually well above 90 per cent. On the eleventh day 6.2 per cent of hydrogen was noted while throughout the remainder of the fermentation covered by the analyses somewhat smaller amounts were found.

The total amount of gas collected from the above fermentation was approximately 69 liters as compared to 99 liters from Vat four and 104 liters from Vat six from the two typical hydrogen fermentations at the same salt concentration.

20° Salometer Brines

Some fermentations resulting from the 20° salting treatment may have small amounts of hydrogen in the evolved gas while in others it may be wholly absent. A typical fermentation at the above salt concentration which demonstrates the first behavior cited is shown in Figure 24. In this case the yeast fermentation started after about the fifth day and continued until the fifteenth day. The gas evolution curve responds with an upward trend during the active growth phase of the yeasts, although compared to other fermentations previously discussed, no great amount of gas was evolved. During the early part of the fermentation (3rd to 9th day), prior to the active gas evolution, it is evident from the gas analyses values that another gas was present since the percentages of carbon dioxide and hydrogen lacked considerable of making 100 per cent. This was probably due to nitrogen from the air bubbles in the cucumber tissue which constituted a portion of the gas sample during the period of slow gas evolution from the vat. However, it is noted that as soon as active evolution started, the percentage of carbon dioxide rose sharply to above 90 per cent and stayed in that range until about the

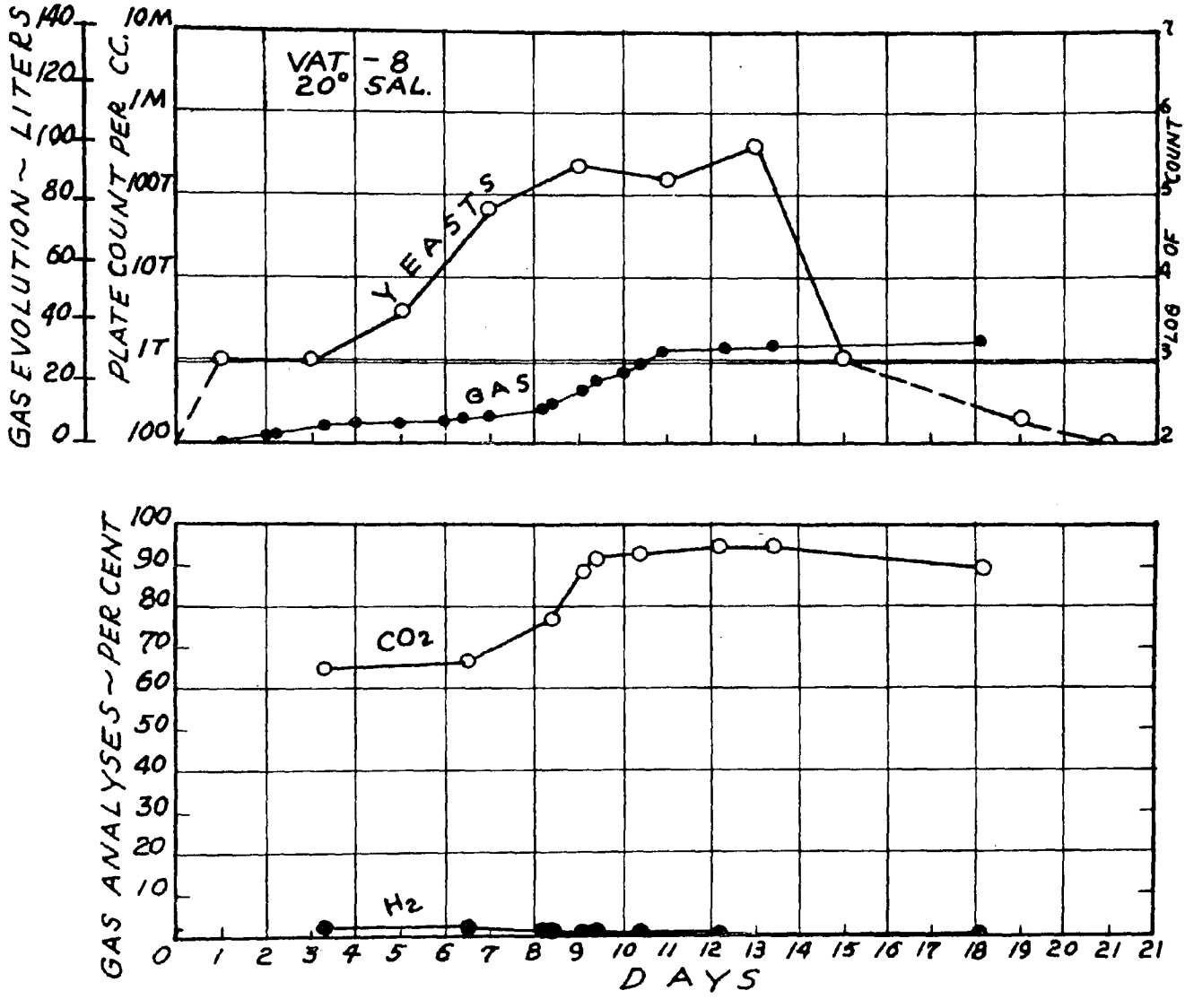

Fig. 24. Yeast populations, gas evolution and analyses of the gas from a fermentation in 20° salometer brine from Vat 8.

eighteenth day. Small amounts of hydrogen were found during the period of analyses. The maximum amount found was two per cent while the major portion of the analyses showed less than one per cent present.

About 32 liters of gas were collected from the above fermentation. This was only about one-half the amount obtained from the 40° fermentation (Vat 10) which showed similar gas analyses values for carbon dioxide and hydrogen. Furthermore, when compared to the gas collected from the active hydrogen fermentations at 40 and 60°, the 20° fermentations showed only about one-third and one-fourth the amount of gas respectively.

The above relationship, of gas production from fermentations at different salt concentrations, is shown in diagramatic form in Figure 25. The final gas evolution values are shown after a collection period of about 26 to 30 days, when no more gas was formed. In general, it is noted that fermentations at the higher salt concentrations resulted in larger quantities of gas. In the 40° series, the two vigorous hydrogen fermentations (Vats 4 and 6) resulted in larger quantities of gas than in the case where this phase of the fermentation proper was inactive (Vat 10).

Several single analyses from additional 40° fermentations are shown in Table 40. These vats were approximately 700 bushel capacity and were put down in the sequence listed in the above table. All developed vigorous gas evolution within about three to five days, and as the gas analyses show, considerable quantities of hydrogen were present in the evolved gas from all fermentations. In general, the results serve to emphasize the preceding data shown for fermentations at this salt concentration (Vats 4 and 6) from which hydrogen was evolved in considerable amounts during the early part of the fermentation.

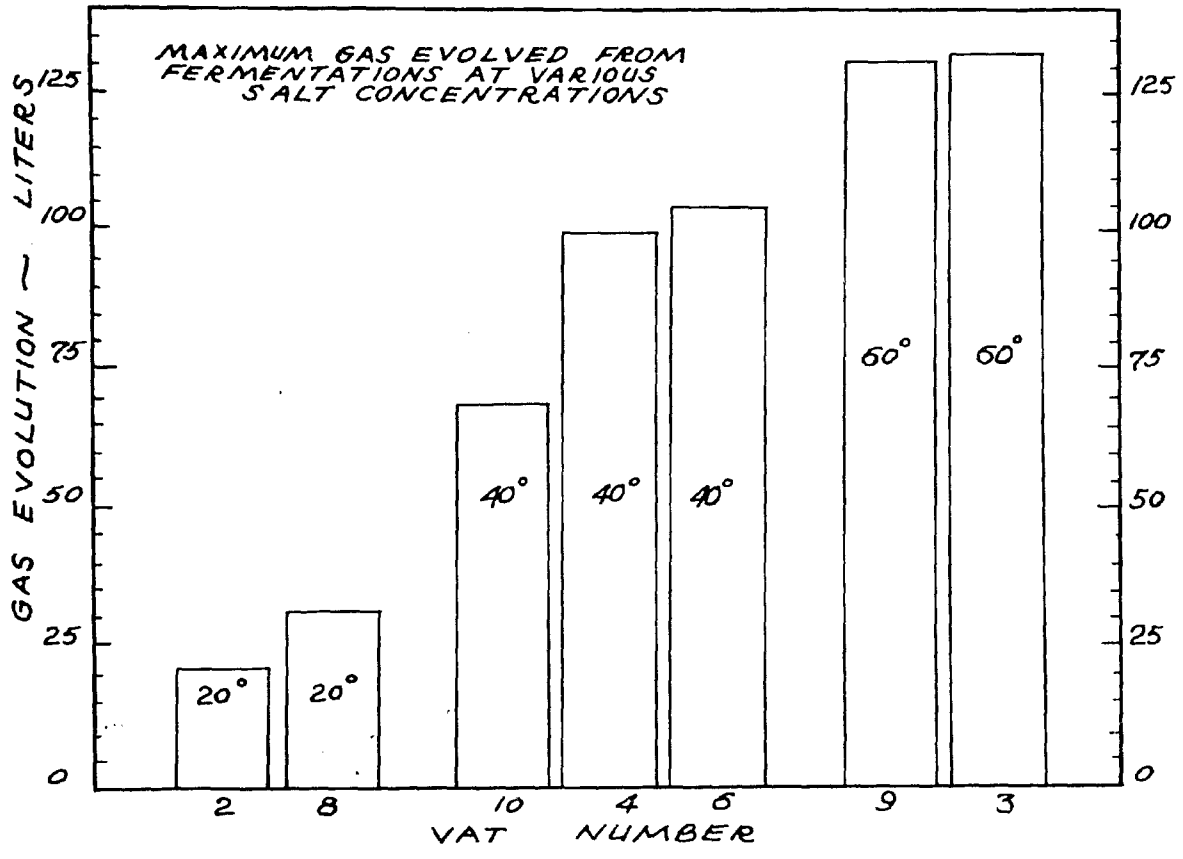

Fig. 25. Effect of salt concentration on the quantity of gas collected from brine fermentations in different vats.

It has been previously mentioned that bloaters or hollow cucumbers were formed during that phase of the fermentation brought about by the organisms belonging to the Aerobacter genus. Due to gas formed within the cucumbers, the latter become distended from gas pressure to such an extent that the three carpels separate and flatten so that often inner cavities of approximately three-quarters the volume of the cucumber are formed. The above condition is brought about more often in the large-sized cucumbers although in some instances even the small sizes are likewise affected.

A comparison of the composition of the gas from typical bloaters with that collected from the surface brine during active hydrogen evolution is shown in Table 41. It is evident from these data that the gas showed no significant difference in composition from either source in any of the three sets of analyses made.

It was found that the above relationship existed only during the short period when actual bloater formation was taking place or at the final point when the bloaters were the most distended due to gas pressure. When the gas from bloaters was analyzed at the conclusion of the active hydrogen evolution period, just prior to the advent of the yeast fermentation, the proportion of hydrogen found was considerably higher. In some instances the increase in the amount of hydrogen was as much as 15 to 20 per cent within two days following active hydrogen evolution from the brine. This condition was presumably due to the greater solubility of carbon dioxide in the brine leaving a greater proportion of the hydrogen inside the cucumbers.

Table 40. Analyses of Gas Evolved from Fermentations in 40° Salometer Brines.

Vat	Age	CO_2	H_2
	days	%	%
R1-T2	7	64.2	35.1
R1-T3	6	62.0	35.7
R1-T4	6	56.8	42.0
R2-T1	5	56.8	42.0
R2-T2	7	55.6	43.8
R2-T3	7	62.2	37.8
R2-T4	6	54.4	45.6

Table 41. Comparison of the Composition of Surface Gas with that from Bloaters or Hollow Cucumbers.

Vat	Age	Surface gas		Bloater gas	
		CO_2	H_2	CO_2	H_2
	days	%	%	%	%
R1-T2	10	62.2	37.0	62.4	36.1
R1-T3	9	64.4	35.2	58.8	39.8
R1-T4	9	58.5	38.5	57.6	38.2

Table 42. Yeast Populations From Fermentations in 20, 40 and 60° Salometer Brines.

Age	20° sal.	40° sal.			60° sal.	
	Vat 8	Vat 10	Vat 4	Vat 6	Vat 3	Vat 9
days	thousands per cc.	thousands per cc.	thousands per cc.	thousands per cc.	thousands per cc.	thousands per cc.
1	1.0	0.6	0.3		0.2	0.6
3	1.0	0.1	0.2	0.1	0.3	0.1
4				0.5		
5	4.0	0.2	0.2	0.6	0.4	0.2
6			3.8			
7	68.0	2.0	0.6	0.5	0.7	0.9
8			1.0	0.9		
9	240.0	1.6	3.3	7.0	2.1	2.9
11	160.0	5.0	1.7	30.0	0.8	0.2
12			1.2	170.0		
13	400.0	1,150.0	5.3	450.0	1.1	1.1
14			18.3	260.0		
15	1.0	210.0	21.0		1.0	1.4
16			1.1	2,480.0		
17		380.0	7.0	1,600.0	21.0	320.0
18			910.0	1,620.0		
19	0.2	210.0	420.0	1,000.0	310.0	12,000.0
20			1,790.0	900.0		
21	0*		400.0	900.0	560.0	2,000.0
22		6.0			1,000.0	156.0
23	0*		260.0	340.0	3,000.0	
24		0.2	14.0	50.0	330.0	125.0
25	0*		2.8	0*	420.0	87.0
26		0*	2.0	0*	520.0	30.0
27			0*			9.0
28			0*		14.0	
29		0*	0*			2.0
30					6.0	0*

* Less than 100 per cc.

Table 43. Gas Evolution from Fermentations in 20, 40 and 60° Salometer Brines.

Age	20° Sal.		40° Sal.			60° Sal.	
	Vat 2	Vat 8	Vat 10	Vat 4	Vat 6	Vat 3	Vat 9
days	liters	liters	liters	liters	liters	liters	liters
1.5	0.9						
2.0		2.2	2.1				1.9
2.5	2.5	3.8			1.0	0.2	
3.5		6.7	3.3		29.0		2.2
4.0	4.6	6.9		1.4			
4.5			3.4	11.6	49.7		
5.0		7.6	4.3	20.1			
5.5	5.0			29.9			
6.0		7.9	5.9	47.4	65.5		
6.5		8.8	6.8	58.8			
7.0	5.5	9.3	7.3	66.8	76.6		
7.5	6.2			77.8			
8.0	7.5	12.0	8.3	83.2	79.3		
8.5	11.2	13.7			85.9		
9.0	14.0	16.8					
9.5		20.7		85.3		0.9	
10.0	18.0	22.3					4.5
10.5		24.7		85.3		2.6	
11.0	21.1	28.4	9.5			4.5	
11.5						7.5	12.8
12.0						10.6	
12.5		30.1	16.0				
13.0	21.7				86.6	20.1	
13.5		31.7	23.3				18.2
14.0			31.0	86.1	89.8	29.1	

Table 43. Gas Evolution from Fermentations in 20, 40 and 60° Salometer Brines (Continued).

Age	20° Sal.		40° Sal.			60° Sal.	
	Vat 2	Vat 8	Vat 10	Vat 4	Vat 6	Vat 3	Vat 9
days	liters	liters	liters	liters	liters	liters	liters
14.5			35.0		92.4		22.2
15.0	22.0		40.9		95.0	35.1	25.0
15.5			45.9		96.8	36.6	28.0
17.0			52.9				32.5
17.5				89.1	98.6		
18.0		32.3	57.9				33.3
18.5	22.1				99.8	39.0	
19.0							43.0
19.5							60.5
20.0							77.4
20.5			65.5	91.6		49.5	88.3
21.0				93.1	102.0	69.5	96.5
21.5					103.1	83.7	
22.0			68.5			95.3	110.3
22.5			68.9	96.3		106.9	
23.0							121.6
23.5				97.7	103.8	115.0	
25.0					103.9	121.5	127.2
25.5				99.1		123.9	
26.0		32.4					
26.5	22.2				103.9		
27.0							131.8
27.5				99.4		128.1	
30.5						132.2	

Table 44. Analyses of Gas from Fermentations in 20, 40 and 60° Salometer Brines.

Age	20° Sal.		40° Sal.						60° Sal.			
	Vat 8		Vat 10		Vat 4		Vat 6		Vat 3		Vat 9	
	CO_2	H_2	CO_2	H_2	CO_2	H_2	CO_2	H_2	CO_2	H_2	CO_2	H_2
days	%	%	%	%	%	%	%	%	%	%	%	%
3							55.0	37.8				
3.3	65.2	2.0										
4							55.5	41.6				
5					50.9	40.6	61.6	36.1				
6					51.7	47.3	55.2	43.2				
6.5	66.9	1.9										
7					50.9	49.4	51.7	47.6				
8[a]	78.2	1.0			51.3	48.1	49.1	50.4				
8.4	86.8	1.0										
9[b]	88.4	0.7					49.0	50.5				
9.4	91.6	0.7										
10.4	92.8	0.5										
11			83.0	6.2					33.8	36.6	32.2	50.6
12[c]	94.6	0.2	91.6	2.4					36.2	49.3	49.4	46.2
13			94.8	1.7					42.9	52.6	50.6	48.5

Footnotes at end of table on next page.

Table 44. Analyses of Gas from Fermentations in 20, 40 and 60° Salometer Brines. (Continued).

Age	20° Sal.		40° Sal.						60° Sal.			
	Vat 8		Vat 10		Vat 4		Vat 6		Vat 3		Vat 9	
	CO_2	H_2	CO_2	H_2	CO_2	H_2	CO_2	H_2	CO_2	H_2	CO_2	H_2
days	%	%	%	%	%	%	%	%	%	%	%	%
13.4	94.6	0.4										
14			96.4	1.5			72.6	24.0	49.6	47.6	55.6	43.2
15			96.2	0.9			81.8	16.0	49.2	48.7	54.0	43.8
16			98.4	0.6					57.0	43.0	56.6	32.4
17			97.0	0.9	63.2	27.4			54.0	35.0	57.8	38.8
18[d*]	89.4	0.3	93.7	0.6					53.6	35.8	73.2	25.4
19			94.7	0.8					54.4	34.9	95.9	3.2
20									63.2	33.1	96.0	2.9
21					81.4	15.6			89.4	8.2	96.9	2.5
22									97.7	1.7	93.2	3.4
23									95.8	2.4	96.8	2.2
24									92.4	3.7	96.0	2.5
25									96.8	2.4	91.8	3.7
26									94.2	3.4	88.7	5.1
28									94.2	3.4		

* For Vat 8 only; a, 8.2 days; b, 9.1 days; c, 12.2 days; d, 18.1 days.
Note: The cases where analyses lack considerable of totaling 100 per cent correlate with periods of slow gas evolution. During this interval the presence of air diffusing from the cucumber tissue constitutes a portion of the sample, thus accounting for the above difference.

SUMMARY AND CONCLUSIONS

Studies upon the microorganisms responsible for the production of hydrogen in cucumber fermentations have been reported. The experimental work has dealt chiefly with the isolation, identification and biochemical studies upon this group of organisms as well as observations upon their typical fermentations under commercial salting conditions.

Twenty-nine cultures were isolated from cucumber fermentations at brine concentrations ranging from 20 to 60° salometer (per cent saturation with respect to salt). Of the above group of cultures, 20 were isolated during the 1938 season and nine during the 1939 season.

The twenty cultures (1938 season) were studied in detail with respect to morphological, cultural and physiological characteristics and based upon this investigation, the organisms were placed in the Aerobacter genus. Eighteen of the 20 cultures that were given detailed study, revealed characteristics in closer conformity to those described in Bergey's Manual for Aerobacter cloacae than for those for Aerobacter aerogenes, the only other species listed. The remaining two cultures (H-138 and 238) are regarded as varieties of Aerobacter cloacae.

A satisfactory apparatus, suitable for studying individual gaseous fermentations with respect to gas production, composition and rate of evolution has been described. The advantages of this type of apparatus are; (a) simplicity of construction, (b) compactness, (c) ease of handling and manipulation, and (d) its relatively small size which makes possible the fermentation of a number of samples in a limited amount of incubator space.

The preliminary experiments dealing with the fermentation of dextrose and cucumber juice by strain H-1438 indicated the following: (a) That the gas produced from these media proved to be composed solely of hydrogen and carbon dioxide; (b) that the composition of the gas from the fermentations depended upon the carbon source, that is, the ratio of hydrogen to carbon dioxide in the gas from the dextrose fermentation was 1:2.3 whereas, from the cucumber juice fermentation it was 1:5.0; (c) that the fermentations for both media were rapid, the major portion of the gas being evolved during the first 48 to 72 hours.

The dextrose fermentations (buffered at pH 5.15) of seven strains from the stock culture collection showed that with five of the group, the amount of gas produced, 139 to 148 cc., was comparable. With the exception of one strain, H-138, the gas evolved from fermentations was similar in composition, the hydrogen to carbon dioxide ratios being between 1:2.32 and 1:2.59. However, in the case of the exception, (H-138), a difference in fermentation behavior was demonstrated not only by a decrease in the amount of gas produced, but also, by a slight increase in the proportion of hydrogen found in the evolved gas (1:1.44). Quadruplicate fermentations of dextrose by the type strain (H-1438) showed no significant differences with respect to gas composition which ranged from 1:2.35 to 1:2.49, H_2 to CO_2. Also, the majority of the fermentations resulted in practically the same amount of evolved gas, 143 to 150 cc.

The fermentation of different lots of dextrose at eight maintained temperatures (5°, 13°, 19°, 24°, 30°, 35°, 40° and 45° C.) showed that the optimum was within the 35° range for maximum gas production and rate of evolution. The lower and higher limitations for the fermentation

were 5° and 45° respectively. Fermentations maintained either above 40° or somewhat below 24° the optimum range of 35° were considerably retarded and less gas was produced. Gas evolution at 19° was much slower than at 40° but at the end of the eight day incubation period, the amount of gas was about the same. The composition of the gas evolved from all fermentations (13° to 40°) was comparable as to percentages of hydrogen and carbon dioxide found.

The fermentation of dextrose took place over a considerable range with respect to initial pH adjustment (pH 5.05 to 9.05) of the medium. However, the fermentation of different lots of dextrose at several buffered pH values (3.6, 4.25, 5.3, 6.0, 7.0, 7.55, 8.05, and 8.85) revealed that pH 5.3 was the optimum as demonstrated by maximum gas production and rate of evolution. pH 3.6 resulted in no growth in the acid range and pH 8.85 seemed to approach the limit of adequate growth in the alkaline range. In general, it was shown by the gas analyses that the percentages of hydrogen and carbon dioxide were similar for all of the above fermentations.

Dextrose fermentations (buffered at 5.15) to which salt was added, to the extent of 5, 10, 15, 20 and 25 per cent saturation, demonstrated a progressive decrease in gas production as the salt concentration increased above five per cent saturation. In this series, salt concentrations as high as 20 and 25 per cent saturation resulted in no growth; a part of the inhibitory effect was due to a lowering of the pH below the optimum (5.3) by the action of salt on the buffered broth. Similar fermentations employing unbuffered cucumber juice resulted in adequate growth with measurable gas evolution in the 20 per cent saturated lot but not

in the 25 per cent saturated lot. There was no significant differences noted as to gas composition between fermentations within the same dextrose and cucumber juice series, although there was a considerable difference in the ratio of hydrogen to carbon dioxide found for the gas evolved from the dextrose lots as compared to that from the cucumber juice lots.

It was demonstrated that certain changes occur during the heating of cucumber juice broth which retards or delays the fermentation as well as decreases the amount of gas evolved. This influence resulted in a much delayed fermentation in juice receiving 100 min. heating (at 15 lb. pressure) as compared to a 40 min. heating although the final volumes of gas after one week were similar.

A comparative study of the composition of the gas evolved from the fermentation of 14 carbon compounds revealed that the proportions of hydrogen and carbon dioxide depended upon the carbon source fermented. The fermentation of l-arabinose, dextrose, d-galactose, levulose, d-mannose and saccharose yielded gas composed of approximately one volume of hydrogen and two volumes of carbon dioxide (1:2). The fermentation of l-xylose, rhamnose, maltose, raffinose, d-mannitol, d-sorbitol and salacin yielded gas of approximately equal volumes of hydrogen and carbon dioxide (1:1). The fermentation of lactose yielded gas composed of approximately two volumes of hydrogen and one volume of carbon dioxide (2:1). In contrast to the results for the above compounds, the fermentation of cucumber juice yielded gas composed of approximately one volume of hydrogen to five volumes of carbon dioxide (1:5).

The study of the typical fermentations brought about by the Aero-

bacter under salting conditions typical of the industry revealed a number of interesting observations. Generally, it was found that the 60° salometer salting treatment showed the most consistent behavior with respect to what is termed the typical hydrogen fermentation. Also, it was observed that the 40° treatment may or may not result in the typical, active hydrogen fermentation. Furthermore, some fermentations resulting from the 20° treatment may have small amounts of hydrogen in the evolved gas while in others it may be completely absent.

The gas evolution as well as the composition of the gas demonstrated that typical fermentations in both 40 and 60° brines were divided into two distinct gas evolution phases; the first was brought about by the Aerobacter group and during the active period of fermentation, the gas was similar in composition with respect to hydrogen and carbon dioxide, the proportion being about 1:1; the second phase was brought about by the yeasts, during which period the gas was composed of practically all carbon dioxide.

In the 40° fermentation, approximately four-fifths of the gas evolved was produced by the organisms of the Aerobacter group while the remainder was contributed by the yeasts. In the 60° fermentation, the situation was somewhat reversed, here approximately one-fourth of the gas evolved was brought about by the Aerobacter while the major portion (three-fourths) was produced by the yeasts.

A comparison of gas evolution from fermentations in 20, 40 and 60° brines revealed that, in general, the fermentations at the higher salt concentrations resulted in larger quantities of evolved gas.

The gas from "bloaters" or hollow cucumbers, that was formed dur-

ing the active phase of the hydrogen fermentation, had practically the same composition with respect to hydrogen and carbon dioxide as did the gas collected from the surface of the brine. However, this relationship only existed during the actual formation period of the bloaters.

Typical yeast fermentations resulted in all brine treatments (20, 40 and 60° salometer) employed for salting cucumbers. The different salt concentrations did not influence the number of yeasts found in the brine, once fermentation was underway. Also, there was no definite correlation between the maximum numbers of yeasts present in brines of different concentration and the amount of gas evolved during their active growth phase.

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