FACTORS AFFECTING THE GROWTH AND VIABILITY OF LACTOBACILLUS ACIDOPHILUS

By

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'11

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INTRODUCTION

The therapeutic value of an acidophilus product has been found to be dependent upon the number of viable organisms at the time of ingestion. An examination of commercial preparations has shown that a majority has failed to meet the specifications of a satisfactory product (James 1927) (Kopeloff and Beerman 1922). Milk cultures are desirable in that the organisms grow readily in milk and the resulting product is usually palatable and nutritious. The necessary number of viable organisms should be present in a properly prepared fresh milk culture; however, any increase in the number of viable acidophilus organisms is desirable.

The purpose of this investigation was to determine some of the factors influencing the growth, viability, and death of Lactobacillus acidophilus with special reference to increasing the number of viable organisms obtainable.

L. acidophilus does not grow well under ordinary conditions like many species of bacteria. Growth is influenced by the nutrient requirements and pH of the medium. Many strains are influenced by the presence of CO_2. Thus, before making quantitative analyses of cultures, a study was made of the influence of these growth factors on various strains.

L. acidophilus belongs to the aciduric group of organisms producing principally lactic acid. This production of lactic acid causes destruction of the viable organisms. Thus an important factor in obtaining the maximum number of viable organisms may be the control of acidity of the medium. Accordingly, the effect of calcium carbonate and phosphate buffering materials on controlling the increased acidity was studied.

HISTORICAL

The literature of the investigations on Lactobacillus acidophilus deals mostly with the isolation, differentiation from other members of the aciduric group, implantation, therapeutic value, and factors affecting growth and cultural studies.

A review of the early work on L. acidophilus is given in the book of Kopeloff (1926) on this subject. Moro (1900) is given credit for the isolation of an organism from the stools of breast-fed infants which he named "Bacillus acidophilus." In the same year Tissier and Finkelstein discovered the organism to confirm his findings. Tissier contended, however, that his B. bifidus was the predominating organism in the stools of breast-fed infants and not acidophilus as claimed by Moro. This finding of B. bifidus, as stated by Kopeloff, was confirmed by Cohn, Rodella, Passini, Jacobson, Bradt and Beifeld, and admitted at a later date by Moro. Logan (1914) concluded that the stools of breast-fed children yielded B. bifidus, while L. acidophilus was found chiefly in stool specimens of artificially fed children. Children fed both breast and bottle milk yielded both types of the lactobacilli.

The confusion of differentiating between members of the genera led to much of the present-day investigations. Rahe (1914) and Kulp and Rettger (1924) proposed a classification of the organisms based upon the fermentation of sugars. Albus and Holm (1928), as well as Day and Gibbs (1928), concluded that fermentation does not offer a means of distinction. Kulp (1929) attempted a separation of L. acidophilus and L. bulgaricus based upon the sensitiveness of indol and phenol. Concentrations inhibitory to L. bulgaricus showed no restraining action upon L. acidophilus, thus showing that it is not illogical to suppose, since these products are found in the intestinal canal of man and animals, that they influence the implantation of the organisms.

Albus and Holm (1925-1926), used surface tension depressants in their medium to study the growth of L. acidophilus and L. bulgaricus, and found that this offered a valuable means of differentiation. In 1927 Kopeloff and Bierman published results to substantiate these findings, thus strengthening the logic of implanting the organisms in the intestinal canal.

Torrey (1915) was one of the first investigators to demonstrate the importance of L. acidophilus. Working with typhoid patients, he found that the addition of 250 to 300 grams of lactose was responsible for the establishment of L. acidophilus as the predominant organism, the degree of transformation being dependent largely upon the type of flora present at the onset of the disease. Reichart and Davis (1928) in summarizing Kopeloff's (1928) work, which covered a large enough number of cases to eliminate unusual or exceptional ones, seems to have established the following: (1) that L. acidophilus was recovered from patients receiving large numbers of these organisms; (2) L. bulgaricus was not recovered from the feces of patients, indicating that it can rarely, if ever, be implanted in the human intestine; (3) constipation is markedly alleviated by the administration of L. acidophilus milk containing two hundred million viable organisms per c.c. and a slight increase in defecation of non-constipated patients; (4) the action of L. acidophilus is not a physical or chemical but a bacterial phenomenon. From a practical standpoint, it has been shown that constipation, diarrhea, and other intestinal disorders may be satisfactorily treated with L. acidophilus.

To meet the difficulty of growing L. acidophilus, various media have been suggested from time to time. One of the first developed was the whey agar proposed by Rettger and Cheplin (1912). Later Rettger and Kulp (1922) found whey broth and whey agar of much value in the isolation and cultivation of L. acidophilus. Because of the exacting technique to be used in obtaining a clear and uniform whey, carbohydrates have been used as substitutes for whey. Galactose agar containing 0.5 to 1.0 per cent of the carbohydrate has served

as a good substitute for whey agar in plating of fecal specimens, in checking purity of cultures, and in isolation of L. acidophilus. Kulp (1923) used casein digest media for the successful growing of L. bulgaricus, obtaining better growth than with commercial peptone media. One hundred c.c. of digest, representing 10 grams of original casein, plus 3 grams of commercial meat extract were used as a base for each liter of medium desired. For routine study and isolation work a digest made from "Klim" (powdered skim milk) prepared in the same manner as casein digest gave excellent results. Kulp and Rettger (1924) in a further study of L. acidophilus and L. bulgaricus obtained results to support their earlier findings, that casein and Klim digest agar was superior to ordinary media; also that galactose was apparently the most favorable carbohydrate. Hunter (1924), in a preliminary report of media for the cultivation of the lactobacillus group, reported best results with a casein digest medium containing one per cent sodium oleate or sodium ricinoleate and sodium glycocholate. The greater growth was attributed to lowering of the surface tension permitting surface growth, and adding something to the medium which the organisms would utilize.

Later investigations by Kulp (1927) in a study of the effect of adding tomato juice to whey-galactose agar found that it gave larger and more characteristic colonies of lactobacillus. Two or four hundred c.c. of tomato juice per liter plus one per cent peptone produced the most satisfactory growth. No satisfactory explanation was offered for the growth stimulating effect other than an "accessory substance or substances." Incubation in an atmosphere containing approximately 10 per cent CO_2 was found desirable for agar platings of both L. acidophilus and L. bulgaricus. In 1932, Kulp and Whit modified this original tomato juice medium by the addition of one per cent peptonized milk which has proved more satisfactory because of larger colony development and usually higher quantitative counts as compared with the original tomato juice agar and the more complicated digest media. Confirmation

was again made that incubation of agar plates in an atmosphere of 10 per cent CO_2 is desirable.

Among the more recent media developed are those of Wallgren and Smith (1932). A digested artificial medium and a tomato digest medium based on the analysis of human colostrum was developed and recommended for the study of the cultural and the morphological changes of the lactobacillus group.

Further recommendations on the use of vegetable peptone agars for quantitative work with L. acidophilus have been reported by Bachmann and Frost (1932). Using a plating agar prepared from an extract of cabbage, quantitative counts were obtained which were higher with consistant regularity as compared to whey-galactose agar, especially, with older milk cultures. As stated by the authors, there may be substances in the cabbage which stimulate the older bacterial cells to vigorous growth. Results with green beans and spinach agars were found to be less favorable as compared to carrots, tomato, and cabbage agars.

Valley and Rettger (1925) in a study of influence of CO_2 on bacterial growth found a complete inhibition of L. acidophilus growth with a complete removal of CO_2 from the surrounding atmosphere. On the other hand, there was an increased growth with increase in CO_2 up to 20-25 per cent. These results are in accord with those of Reichart and Davis (1928) who found an increase in viable numbers in an atmosphere up to 8 per cent CO_2.

Roos (1926) in a study of cultural characteristics of L. acidophilus concluded that this name should be applied to a group of biologically related strains, variable in cultural and morphological characteristics. Kopeloff (1934) states in his comment on dissociation of L. acidophilus that the evidence indicates it is the R strains (rough) of intestinal origin which have therapeutic values. Transitions from the R to S strains have been reported, but no authentic reports have been made of transition from S to R strains. This fact tends to place strains of intestinal origin apart from

strains of lactobacillus of dental origin.

On the basis of colony formation as many as 5 different strains have been isolated from one specimen. Smith, Gottschall and Wallgren (1932) also concluded that the morphological and cultural characteristics of L. acidophilus depend upon such factors as food supply, the pH value of the medium and the presence of small quantities of inorganic metals, such as zinc, copper, iron, etc., and may be converted to assume the characteristics of other members of the group.

In studying the effect of acidity on lactobacillus cultures Black (1931) reported that with the strains used the highest plate counts were obtained when the acidity of culture reached approximately 1 per cent. At higher acidities a destruction of organisms took place, which also varied with the strains.

A complete review of the literature has revealed no evidence of the use of buffers in controling the acidity of L. acidophilus cultures.

EXPERIMENTAL

METHODS

Cultures: The cultures used in the following experiments were secured from our stock cultures or from the collections of other investigators designated to be Lactobacillus acidophilus. (Page 7).

All transfers of cultures were made to tubes of sterile litmus milk. Stock cultures were transferred every two weeks. After coagulation the tubes were placed at refrigerator temperature for storage. Preceding their use transfers were made at twenty-four hour intervals for several days in order to obtain an actively growing culture.

Media: The medium used for plating throughout these experiments was tomato juice agar. Preparation was according to the procedure recommended by Kulp and White (1932). Mixture A: Ten grams of Difco peptone and 10 grams of peptonized milk were added to 400 c.c. of tomato juice filtered from a good quality of canned tomatoes. This mixture was heated gently in flowing steam to dissolve the peptone and peptonized milk. Unnecessary heating of the tomato juice was to be avoided because of the darkening of the final product. Mixture B: Fifteen grams of dried agar were added to 600 c.c. of distilled water and heated in the autoclave until completely dissolved. Just previous to removal from the autoclave, Mixture A was heated to near boiling temperature. Mixtures A and B were mixed while hot and filtered through a thin layer of absorbent cotton. The filtered medium was distributed into flasks and sterilized at 15 pounds pressure for twenty-five minutes. The prepared medium was a clear agar of a light brown color. Because of the difficulty encountered with spreaders, due to moisture on the surface of the plates, 1.5 per cent agar was found to be more satisfactory

Culture	Colony Type		Source
22	Rough	Curran	rat feces
42	"	"	acidophilus milk
34	"	"	Prucha (his #A-17)
49	Smooth	"	Prucha (his #A-17)
64	Rough	"	acidophilus milk Towt. lab. Oakland, Calif.
73	Smooth	"	Calif. Dept. Agric. (from Rettger 1928)
61	"	"	Enright (dental caries #42)
75	"	"	Golden State Milk Prod. Co. San Francisco, Calif. (U. S. B. D. I. #1)
K	"	England	originally from Dr. N. Kopeloff
K4y	"	Kopeloff	single isolation from acidophilus colony from feces of human subject ingesting Rettger's strain acidophilus cultures.
H	"	Kopeloff	single cell isolation from acidophilus colony from feces of human subject ingesting Rettger's strain of acidophilus culture.
R1	Rough	Myers (his #RL8C)	human feces
R2	"	" (his #RL8D)	" "
R3	"	" (his #RL8E)	" "
R4	"	" (his #LA7C)	acidophilus milk
R5	"	" (his #LAR)	" "
I-6	Intermediate	" (his #985R)	" "

than 1.1 per cent as recommended in the original formula.

The reaction of the medium was adjusted before use. This was found to be more satisfactory because of the decided drop in the reaction during sterilization, the final reaction sometimes being as low as 5.2. All reactions were determined by means of quinhydrone electrode and saturated calomel half cell.

Plating: The dilutions were made in six-ounce screw cap glass prescription oval bottles containing 99 c.c. of sterile tap water after sterilization. The use of this type of bottle permitted thorough shaking of the samples, a procedure which is essential for breaking up the coagulated milk. Using 1 c.c. of the original material, dilutions of 1: 1,000,000 and 1: 10,000,000 were found to be satisfactory in a majority of the experiments.

Each dilution was plated in duplicate. The plates were poured with 10-12 c.c. of agar. This was found to be the most satisfactory quantity to insure the correct amount of moisture, thus eliminating the possibility of drying and the appearance of spreaders. The plates were incubated at 37°C. for five days.

The entire plating procedure was planned to shorten, as much as possible, the time between removal of inoculum and pouring of agar. In comparison studies, the plates were so arranged so they could be poured in the shortest possible time.

Counting:

The plates were counted by the aid of the Lumi-lens counting device. Tabulations were made by means of a hand tally.

I. EFFECT OF pH OF THE MEDIUM ON GROWTH OF LACTOBACILLUS ACIDOPHILUS

In reviewing the literature of the various investigations on Lactobacillus acidophilus, it has been noted that the media used in different studies varied in the reaction. Kulp and White (1932) observed that some strains of L. acidophilus which developed poorly on a medium of pH 6.8-7.0 showed satisfactory growth when acidified to pH 6.0-6.2. Smith, Gottschall and Wallgren (1932) found that the cultural characteristic of L. acidophilus varied upon the changing in the pH value of the medium.

Thus, before studying other factors effecting the growth of L. acidophilus it was desirable to find the optimum reaction of the media for the colony development of different cultures.

The cultures used in this investigation were from various sources, and differed in cultural characteristics. Cultures R1, R2, R3, R4, R5, 22, 42, 64 and H were rough strains. Cultures 49, K, K4y, 75 and 73 were smooth strains. I-6 was classed as an intermediate strain.

Tomato juice agar adjusted to the pH values of 5.8; 6.0; 6.2; 6.4 and 6.6 were used for this comparison.

Dilutions of 1: 1,000,000 and 1: 10,000,000 were prepared from milk cultures. Duplicate plates from each dilution were prepared for each pH value of the agar. To remove, as far as possible, the chance of variation in the amount of culture in each plate the same pipette was used for transferring from the dilution bottle to the series of plates. The plating procedure was arranged so that the time elapsed between inoculating the plates and pouring the agar was approximately the same.

Adjustment of the medium to the desired pH value was done just prior to pipetting the dilutions to the plates. All reactions were determined

by means of the quinhydrone electrode and saturated calomel half cell. Adjustments of reactions were made by the addition of sterile tenth normal sodium hydroxide or tenth normal hydrochloric acid.

The plates were inverted after hardening of the agar and incubated at 37°C. for four to five days.

TABLE I

Effect of pH of Medium on the Growth of Lactobacillus Acidophilus
(Counts in Millions per c.c.)

pH 5.8	pH 6.0	pH 6.2	pH 6.4 *
	Culture R		
6.2	126	1.7	4.5
0	315	0	0
584	860	400	16
323	770	615	0
	Culture R2		
41	91	45	73
56	148	322	239
53	148	149	53
380	680	715	395
510	539	567	510
	Culture R3		
253	250	260	236
39	253	346	0
490	793	797	361
	Culture R4		
1.3	214	27	2.2
53	95	31	5
785	1310	577	223
895	1525	1015	810
173	505	298	41

pH 5.8	pH 6.0	pH 6.2	pH 6.4
	Culture R5		
290	363	338	316
167	283	307	121
815	970	1320	665
328	312	331	222
	279	150	38
	Culture H		
740	955	885	710
785	905	720	400
261	348	329	264
	Culture 22		
6	88	96	57
725	690	760	600
325	395	305	215
463	438	442	454
	Culture 42		
57	765	720	318
515	605	605	465
269	370	379	240
35	222	230	191

* pH 6.6. Results omitted from table; however, the results were always lower than pH 6.4.

TABLE I (Cont'd)

Effect of pH of Medium on the Growth of Lactobacillus Acidophilus
(Counts in Millions per c.c.)

pH 5.8	pH 6.0	pH 6.2	pH 6.4
	Culture 64		
668	593	674	557
676	900	915	870
399	501	514	183
765	1055	1135	1100
	Culture K		
179	209	442	152
403	354	812	352
259	226	416	705
120	112	157	155
222	219	239	200
	Culture K4y		
1350	1445	1495	1065
190	187	226	215
715	735	775	630
	Culture 49		
621	578	546	604
562	637	555	581
799	819	859	884
801	802	796	787
670	765	600	640

pH 5.8	pH 6.0	pH 6.2	pH 6.4
	Culture 73		
581	588	590	600
620	587	577	532
879	846	918	880
880	842	942	940
	Culture 75		
3181	326	332	278
490	650	975	590
715	745	785	775
	Culture I-6		
151	167	162	143
319	306	303	311
675	810	790	805
369	334	335	335

Discussion

According to the results of duplicate plates presented in Table I the optimum reaction for all cultures studied were in the range of pH 6.0-6.2. The counts in nearly all cases were higher in this average range than at pH 5.8 or 6.4. The rough strains R1, R2, R3, R4, R5, H, 22, 42 and 64 were limited more to this range than the smooth strains which in many instances attained as high count when the reaction was adjusted to 5.8 or 6.4. In no instances were the counts higher at pH 6.6. Strains R1 and R4, which showed poor colony development in all cases, were especially limited to pH 6.0.

Plates of the series which showed the highest counts were also characterized by an increase in the size of the colonies compared to those of lower counts. This increase in colony development removed the difficulty encountered in counting with some strains.

II. COMPARISON OF MEDIA FOR DETERMINING VIABLE NUMBER OF LACTOBACILLUS ACIDOPHILUS

Lactobacillus acidophilus does not grow well on plain nutrient agar. To meet this difficulty, various media have been developed from time to time. The most widely used are: the whey agar proposed by Rettger and Cheplin, (1912); the galactose agar of Rettger and Kulp (1922); the casein digest-galactose agar of Rettger and Kulp (1924); the whey peptone galactose agar of Kulp (1926); and the tomato juice agar of Kulp (1932). The whey galactose peptone agar and tomato juice agar are made in the dehydrated form. Further investigations have indicated that strains of Lactobacillus acidophilus vary in their ability of colony development on various media.

A. Comparison of Whey and Tomato Juice Agars: Since whey agar and tomato juice agar are widely used in quantitative analysis of Lactobacillus acidophilus cultures, a comparative study was made of the ability of some strains to develop colonies on the two media. For this study cultures K, 49, 54, 60, 64, 73, 74 and 75 were used.

Dilutions of 1: 1,000,000 and 1: 10,000,000 were prepared from milk cultures. From each dilution quadruplicate plates were prepared, one set of duplicates being poured with tomato juice agar and the second being set with Bacto dehydrated whey agar. The reaction of the media was adjusted to pH 6.2 which was found to be the optimum reaction. The plating procedure was arranged so that the time between inoculation of the plates and pouring the agar was approximately the same. After solidification of the agar, the plates were inverted and placed at 37°C. without an increase of CO_2 in the surrounding atmosphere. Incubation was from 4 to 5 days.

B. Comparison of Tomato Juice, Wheytone and Kraftose Agars: Two new milk powder products (Wheytone and Kraftose) have been developed. Both of these products are sweet whey powders. Wheytone product is enriched by the

addition of the milk minerals calcium and phosphates.

Because of the difficulty of preparing whey in small quantities and the similarity of these products to other whey preparations, agars were prepared from each to determine their value as a plating medium for L. acidophilus cultures. Preparation of the Wheytone agar and Kraftose agar was based on the analysis of whey. Fifty-two grams of the powder were added to 1000 c.c. of distilled water and heated in flowing steam for 3 hours. After heating, the precipitate was removed by filtration through a thin layer of absorbent cotton, followed by filtration through filter paper. One per cent (Difco) peptone and 1.5 per cent dried agar were added to the clear filtrate, heated until completely dissolved and refiltered through a thin layer of absorbent cotton. After distribution into flasks, the medium was sterilized at 15 pounds pressure for 25 minutes. The resulting media were clear agars of light color.

Cultures R2, R3, 75, 22, H, K4y, and I-6 were used in this study. Tomato juice agar was used as the control medium for making the comparative counts. The procedure for preparation of dilutions, plating, and titration of the media was the same as that in the quantitative comparison of tomato juice agar and whey agar.

TABLE II

Comparison of the Counts Obtained with Tomato Juice Agar and Bacto Dehydrated Whey Agar

(Counts in Millions)

Culture K		Culture 49		Culture 54		Culture 60		Culture 64		Culture 73		Culture 74		Culture 75	
Tomato	Whey	Tomato	Whey	Tomato	Whey	Tomato	Whey	Tomato	Whey	Tomato	Whey	Tomato	Whey	Tomato	Whey
442	416	238	120	491	501	151	165	96	100	47	44	306	214	440	428
1,700	1,940	1,560	1,500	74	60	365	360	2,190	2,380	656	555	1,240	1,000	138	140
2,080	2,050	283	246	269	260	370	351	2,050	1,890	236	227	643	630	335	326
1,760	1,070	947	679	247	259	641	620			222	191	1,460	1,640	343	360
1,660	1,590	3,060	2,900			1,360	1,290			505	750			721	681
452	405	1,870	2,320							630	435				
209	226	818	801							740	760				

TABLE III

Comparison of the Counts Obtained with Tomato Juice Agar, Kraftose Agar and Wheytone Agar

(Counts in 1,000,000)

Culture R2			Culture R3			Culture 75			Culture 22			Culture H			Culture K4y			Culture I6		
A*	B*	C*	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
470	613	800	373	436	429	650	420	580	638	666	548	197	187	258	1,305	1,400	1,390	178	181	207
193	241	330	325	1,015	600	1,170	1,215	1,630	395	790	610	124	109	149	1,855	1,780	1,835	180	156	170
33	151	166				571	560	530	295		305	242	229	237	935		920	634	593	629

* A - tomato juice agar
B - Wheytone agar
C - Kraftose agar

Discussion

The results of comparative plating of acidophilus in tomato juice agar and Bacto dehydrated whey agar are incorporated in Table II. In this and Table III the counts are averages of duplicate plates expressed in millions but the 6 ciphers are always omitted.

According to the results of this experiment, as shown in Table II, there is little difference in the counts obtained with tomato juice agar and dehydrated whey agar. However, the colonies in tomato juice agar attained a larger development in size than in whey agar. This increase in the size of colonies made counting less difficult. It was also observed that colonies from rough strains were more filamentous in their development on whey agar than on tomato juice agar.

In Table III are recorded the counts obtained with tomato juice, Kraftose and Wheytone agars. According to the results as found in this table, there was no appreciable difference in the counts obtained with the three media from cultures 75,22, H, K4y and I-6. However, from cultures R2 and R3 a decided higher count was obtained with Wheytone agar and Kraftose agar. The lower counts obtained with tomato juice agar were associated with the diffuse flat and gray colony of cultures R2 and R3 on this media which made counting difficult. It was necessary to resort to the use of transmitted light as an aid in counting. In Wheytone agar and Kraftose agar the colonies were more compact and could be easily distinguished in the clear media.

Thus, from the results obtained in this comparison study of media, it was found that, with the cultures used, dehydrated Bacto whey agar and tomato juice agar gave approximately the same counts. Tomato juice agar has advantages since the colonies of many strains are decidedly larger than on whey agar.

Media prepared from the sweet milk powders Wheytone and Kraftose were clear plating agars and were found to be satisfactory for the plating of

III. EFFECT OF CARBON DIOXIDE ON THE GROWTH OF LACTOBACILLUS ACIDOPHILUS ON AGAR PLATES

In previous investigations it was observed that there were variations in the development of colonies from different cultures of Lactobacillus acidophilus. Some strains showed only meager colony development on the surface of the agar, other strains failed to show surface development.

Kulp (1927) (1932) observed that CO_2 was desirable for the development of Lactobacillus acidophilus colonies. Valley and Rettger (1927) have shown that CO_2 is necessary for development of the organisms studied and that while many species will grow and multiply normally in the presence of atmospheric CO_2, others are accelerated in their growth when incubated in an atmosphere of 1 to 10 per cent CO_2. Lactobacilli were found to belong to this latter group.

Thus, the purpose of this study was to determine the influence of varying percentages of carbon dioxide on colony development.

The cultures of L. acidophilus used in this investigation were strains 49, 75, 73, K, and K4y, R1, R2, R3, R4, R5, 22, 42, 64, and H.

Three sets of duplicate plates were prepared from 1: 1,000,000 and 1:10,000,000 dilutions of milk cultures. One set of duplicates was placed in an atmosphere of 10 per cent CO_2 and a second set in an atmosphere of 20 per cent CO_2. The third set of duplicates was incubated without an increase in CO_2 above that of the normal atmosphere.

The CO_2 chambers used were round museum jars equipped with the two-hold type lid. One hole rubber stoppers fitted with glass tubing served as inlets for the CO_2 gas and outlets for the escape of air from the chamber. The plates were placed in the chamber and the lid clamped on tightly. The

CO_2 inlet was attached to a cylinder of compressed CO_2 gas. The outlet from the chamber was connected to a wash-bottle filled with water. Connections were made by means of rubber tubing. The CO_2 was allowed to enter the chamber, forcing a corresponding amount of water from the bottle. The volume of air in the chamber being previously determined, it was found by analysis that the volume of water displaced from the bottle was a direct measure of the CO_2 entering the chamber. After the desired amount of CO_2 had entered, the connections were closed by means of clamps. The containers were then incubated as usual.

The reaction of the tomato juice agar used throughout this investigation was adjusted to the optimum for all cultures as previously determined. The plates were not inverted when placed in the CO_2 chamber, because of the difficulty encountered with spreaders.

TABLE IV.

Effect of Carbon Dioxide on the Growth of Lactobacillus Acidophilus

(Counts in Millions per c.c.)

Control	Carbon Dioxide	
	10 per cent	20 per cent
	Culture R	
122	1240	1285
57	182	163
420	635	610
	Culture R2	
233	311	320
465	446	463
285	745	840
	Culture R3	
361	453	520
325	455	505
195	335	335
373	490	476
	Culture R4	
577	700	810
177	473	307
203	312	314
286	419	439

Control	Carbon Dioxide	
	10 per cent	20 per cent
	Culture R3	
150	265	255
139	297	213
346	510	410
331	334	364
340	665	645
	Culture H	
470	785	765
329	329	302
266	280	407
540	575	435
	Culture 22	
202	600	530
146	287	176
	Culture 42	
379	360	271
230	219	205
805	362	405

TABLE IV (Cont'd)

Effect of Carbon Dioxide on the Growth of Lactobacillus Acidophilus
(Counts in Millions per c.c.)

Control	Carbon Dioxide	
	10 per cent	20 per cent
Culture 64		
890	920	985
1180	1280	1360
685	681	601
Culture K		
2060	1780	1970
365	360	366
2890	2750	2510
1680	1450	1365
242	258	244
Culture K4y		
226	204	176
1260	1280	1205
1210		945
Culture 49		
395	430	421
1620	1450	1440
275	254	257
220	202	243
242	177	195

Control	Carbon Dioxide	
	10 per cent	20 per cent
Culture 73		
1075	1045	975
395	430	421
815	855	850
1835	1745	1795
Culture 75		
650	600	525
1170	1320	1415
505		475
Culture I-6		
302	259	258
61	56	50
510	540	585

Discussion

The results given in Table IV show that some strains of *Lactobacillus acidophilus* show higher counts when incubated in an increased percentage of CO_2. This increase was with the rough strains R1, R2, R3, R4, R5, and 22. The rough strains H, 42, and 64, or smooth strains 49, K, K4y, 73, and 75 apparently were not influenced by the increased presence of CO_2.

An atmosphere of 20 per cent CO_2 did not increase the count over that of 10 per cent CO_2. Thus, it would appear that a CO_2 tension of over 10 per cent would not be necessary.

It was observed that the size of the colonies from cultures R3, R4, R5, and 64 were larger when incubated in an atmosphere of 10 per cent and 20 per cent CO_2. There was a tendency of some rough cultures to show development of smooth colonies on the surface of the agar in an increased CO_2 tension.

IV. DISSOCIATION STUDIES WITH LACTOBACILLUS ACIDOPHILUS

Smooth and rough variants in Lactobacillus acidophilus have been observed by various investigators. In certain other strains of bacteria, it is possible to change from rough (R) to smooth (S) and S to R. However, with Lactobacillus acidophilus of intestinal origin, variations from R to S have often been reported, but variations from S to R are exceptional. This was shown by the unsuccessful experiments of Kopeloff (1934) who used marginal fishings from old cultures, exposure to bacteriophage and exposure to immune serum in attempting to force dissociation.

Hadley's (1930) description of S colonies as smooth, regular, and convex; R colonies as rough, irregular and flat, is probably the most typical explanation that can be given. Roos (1926) described a typical adidophilus colony as one with fuzzy edges, (when observed under the low power objective), and this form has been designated as Type X, and colonies showing only a few hair-line projections designated as Type Y. Kopeloff (1934) designates a typical R colony as one with distinct radiating threads of unequal length, visible to the maked eye when grown from three to seven days on casein digest agar. A smooth colony is round or elliptic with an entire edge. However, many strains may fall between these two extremes.

In general it is believed that the typical R strains of intestinal origin are most beneficial for therapeutic purposes. The S strains are considered by some to be of dental origin.

Smith, Gottschall, and Wallgren (1932) studying the cultural characteristics of lactobacillus observed changes in the colony morphology. Variants were found to depend upon the nature of the food supply, the pH of the medium, and the presence of small quantities of inorganic metals, such as iron, zinc, and copper.

It was observed in the previous experiments that plates from rough strains of lactobacillus showed variations in colony formation if incubated in 20 per cent CO_2 atmosphere. These variations were characterized by the development of smoother colonies on the surface of tomato juice agar plates.

The predominant surface colonies gave the appearance of a rough colony protruding thru the surface of the agar in the form of secondary growth. This secondary growth was convex with an entire margin. Other variants appeared as small, thin, flat colonies with an entire margin, showing a granular appearance in the center. A third distinct form might be described as a small compact, rough colony overgrown by a large smooth and flat colony. Colonies beneath the surface of the agar appeared as typical. These dissociated forms of surface colonies developed from cultures of intermediate and rough strains.

Strains used in this investigation were isolated from plates of the rough cultures R2, R3, R4, R5, and H. Isolations made from cultures R2 and R3 failed to grow when transferred to milk. Culture I-6 was used as the intermediate strain.

The technique used throughout this work was essentially the same for each strain. Marginal fishings were made by the use of a straight transfer needle. Transfers were made to tubes of litmus milk and incubated at 37°C. until coagulation appeared. The media used for plating was tomato juice agar, prepared as previously described for other investigations. A sufficient range of dilutions were prepared to insure well-isolated colonies on the plates. The plates for each dilution were then poured in quadruplicate, one set of duplicates was placed in the CO_2 chamber and 20% of gas allowed to enter, the second set remained in atmospheric air to be used as controls. Incubation was, as usual, at 37°C. from four to five days. Colony morphology studies and isolations were aided by the use of a binocular dissecting microscope and low power objective.

To begin this study, isolations were made from the secondary -like growth and from the smoothest of the surface colonies of plates which had developed in a 20 per cent CO_2 atmosphere. Isolations and platings were repeated from these variants six times.

Discussion

Examination of plates from the first isolations showed no variations in the type of colony on the control plates.

Colonies growing beneath the surface of the agar appeared similar to those of the control plate. When observed under the dissecting microscope a more compact growth was observed in the center of the colony.

No apparent increase in transition could be observed in the second platings over that of the first platings. Examination of colonies resulting from the third and fourth platings showed that the colonies of the control plates were smaller with more compact centers. The radiating filaments were shorter and thicker, giving the appearance of an intermediate type of colony (Plate IX). Surface colonies developing under CO_2 were the same as previously described (Plates V, VI, VII, XI). The subsurface colonies were smaller and compact. (Plate VIII).

Strain I-6, which is considered an intermediate (Plate IV) revealed more variation when subjected to same environmental conditions. The surface colonies after the first transfer were similar to those of the smooth strain K4y (Plate I). Colonies developing in the medium also showed characteristics of being smooth when examined with the naked eye. Further examination with the aid of the dissecting microscope and low power objective revealed small hair-like filaments radiating from the lens-shaped colonies. (Plate IX). Irregular forms of compact colonies with long interlacing filaments were also observed. (Plate X).

Isolations and platings were repeated for the fifth time and showed no further variation.

Plate I. *L. acidophilus.* Smooth surface colony on tomato juice agar incubated in atmospheric air.

Plate II. *L. acidophilus.* Smooth subsurface colony in tomato juice agar incubated in atmospheric air.

Plate III. *L. acidophilus.* Rough subsurface colony in tomato juice agar incubated in atmospheric air.

Plate IV. *L. acidophilus.* Intermediate subsurface colony in tomato juice agar incubated in atmospheric air.

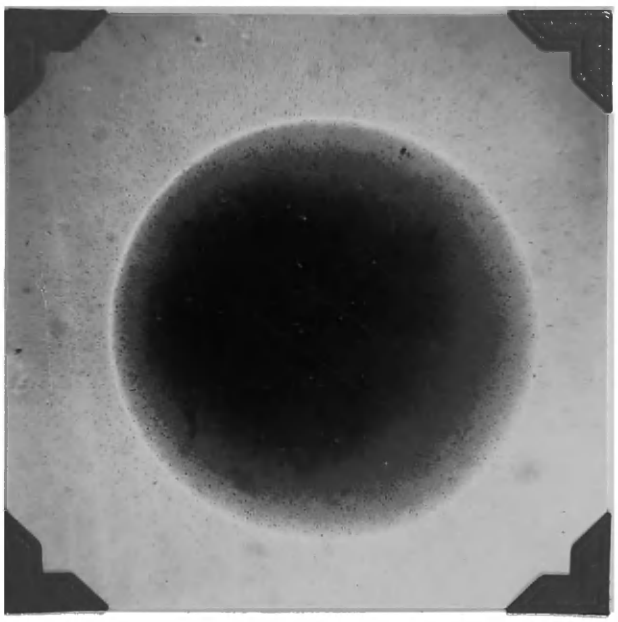


PLATE I

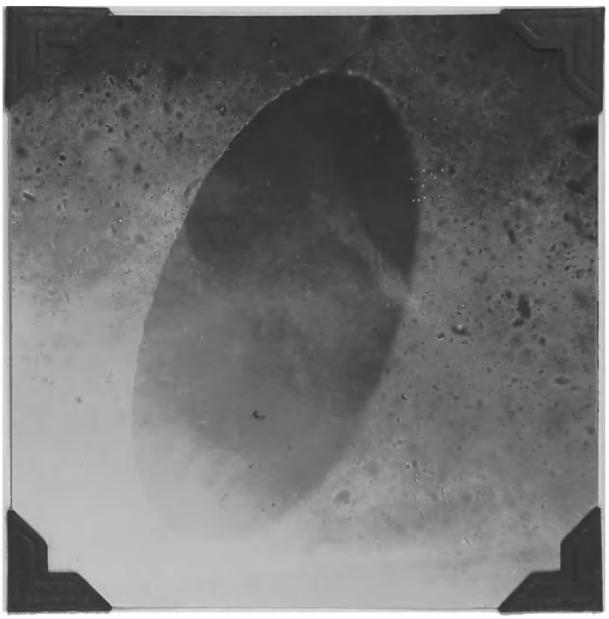
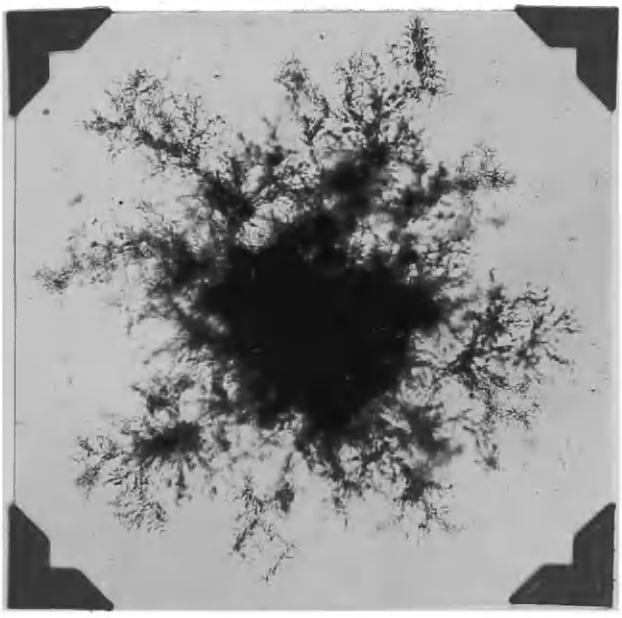


PLATE II



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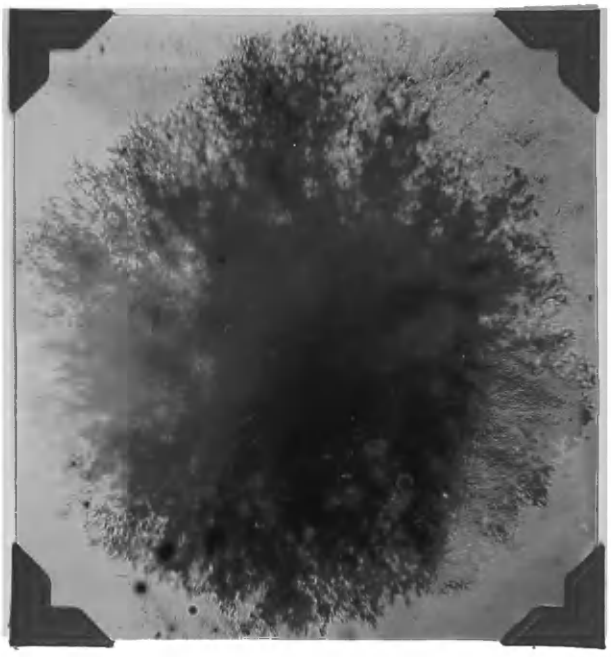

PLATE IV

Plate V. *L. acidophilus*. Surface colony of rough strain on tomato juice agar incubated in an atmosphere of 20 per cent CO_2.

Plate VI. *L. acidophilus*. Surface colony of rough strain on tomato juice agar incubated in an atmosphere of 20 per cent CO_2.

Plate VII. *L. acidophilus*. Surface colony of rough strain on tomato juice agar incubated in an atmosphere of 20 per cent CO_2.

Plate VIII. *L. acidophilus*. Subsurface colony of rough strain in tomato juice agar incubated in an atmosphere of 20 per cent CO_2.

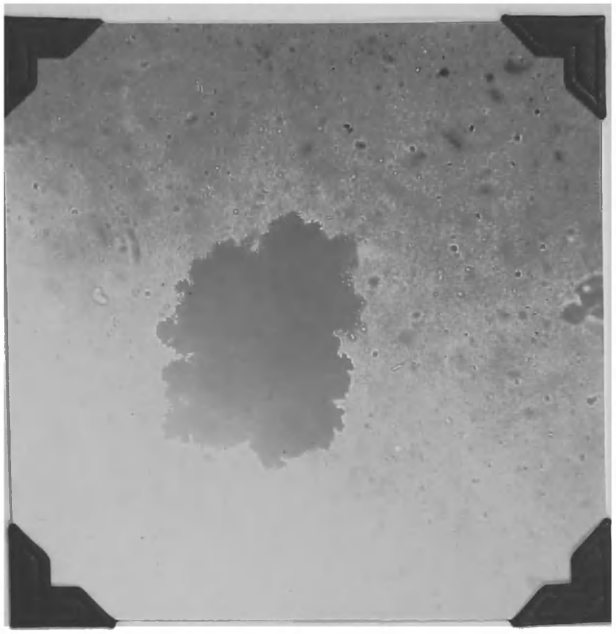

PLATE V

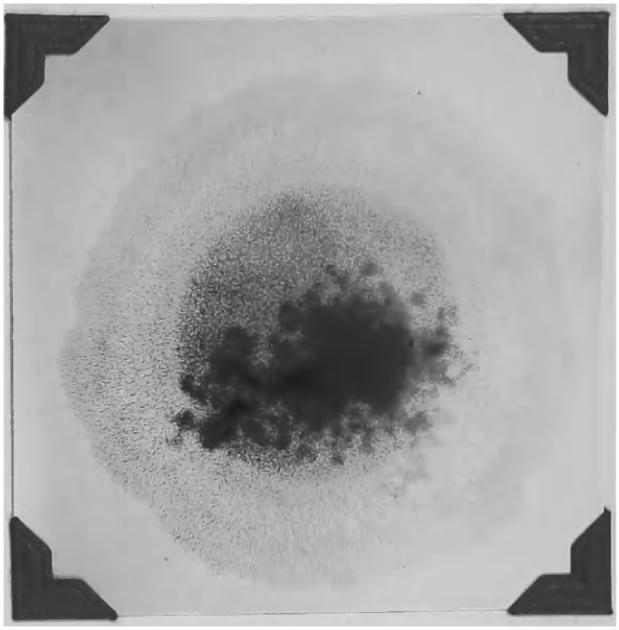

PLATE VI

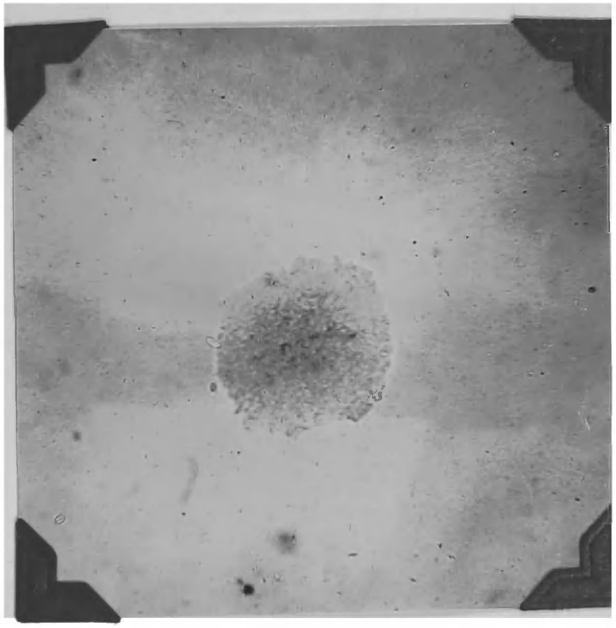

PLATE VII

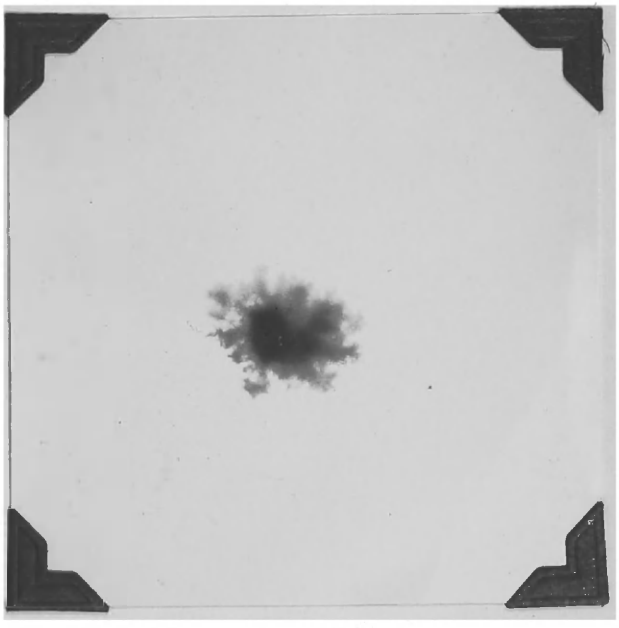

PLATE VIII

Plate IX. *L. acidophilus.* Intermediate subsurface colony in tomato juice agar incubated in an atmosphere of 20 per cent CO_2.

Plate X. *L. acidophilus.* Rough subsurface colony in tomato juice agar incubated in an atmosphere of 20 per cent CO_2.

Plate XI. *L. acidophilus.* Intermediate surface colony on tomato juice agar incubated in an atmosphere of 20 per cent CO_2.

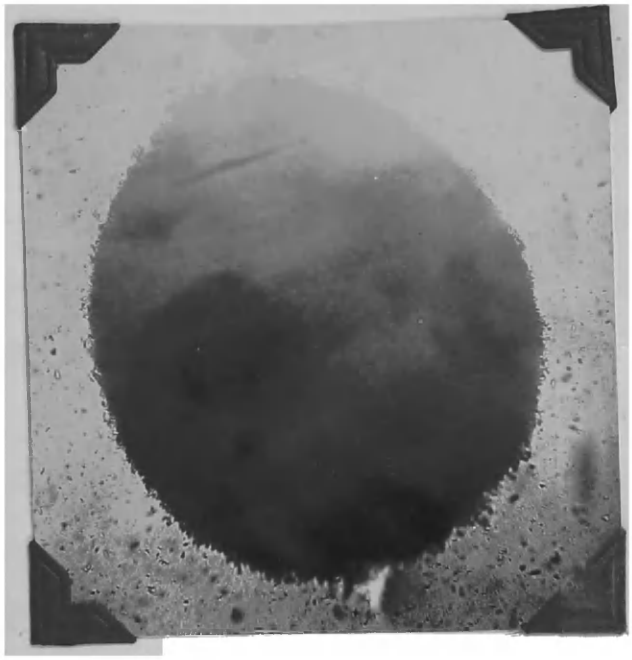

PLATE IX

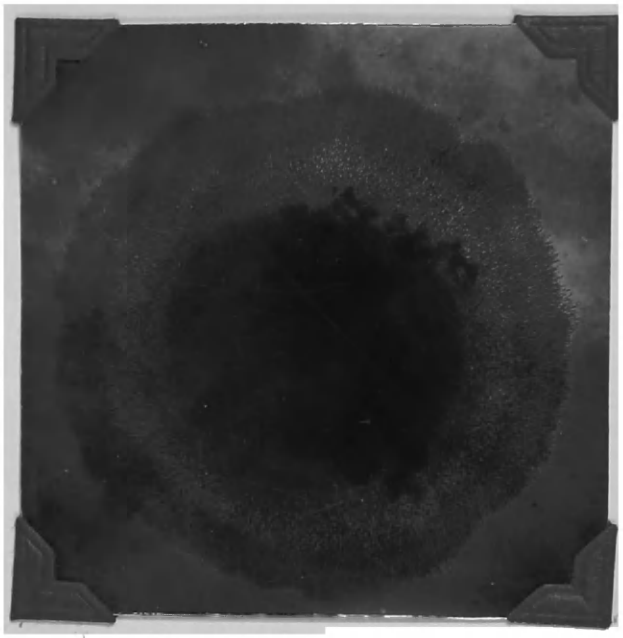

PLATE XI

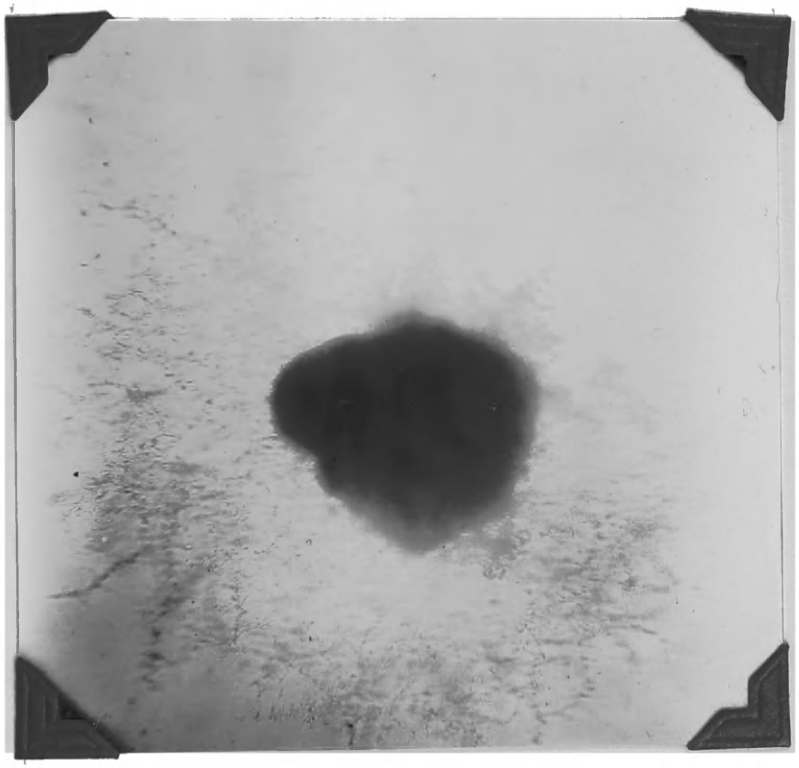

PLATE X

V. EFFECT OF BUFFERING SUBSTANCES ON THE GROWTH OF LACTOBACILLUS ACIDOPHILUS

The therapeutic value of an acidophilus product has been definitely connected with the number of viable organisms at the time of ingestion. Thus, any factors which may be employed to increase the number of viable organisms is desirable.

Increases in the acidity of acidophilus cultures has been found to be responsible for decreases in the number of viable organisms. Thus, the addition of substances to the medium which would retard an increase in acidity and lowering of reaction, should furnish conditions for continued growth. It was the purpose of this work to determine the effect of calcium carbonate and a phosphate buffer toward increasing the number of organisms in a culture. The cultures used in the experiment were R2, R5, 42, H, 49, K, K4y, 73 and I-6. As previously stated, all cultures were grown in sterile skim milk and transferred at 24-hour intervals for a few days before use.

The salts used in the phosphate buffer were NaH_2PO_4 (C. P.) and K_2HPO_4 (C. P.). Fifty per cent solutions of each were made in distilled water and sterilized at 15 pounds pressure for 25 minutes. Sufficient amounts of each solution were added to the media to give 1 per cent and 2 per cent of total salts. Sterile skim milk prepared at different times was found to vary in buffering action. Thus, it was necessary to determine the proportion of salts mixtures for each supply of milk. The final reaction of the milk was buffered to pH 6.2.

Twelve and one-half gram quantities of calcium carbonate (Baker's C. P.) were placed in tubes and heated at 180°C. for three hours to insure sterility. Sufficient quantities were added to give the desired percentage.

250 c.c. of media was placed in 500 c.c. flasks and sterilized at 15 pounds pressure for 25 minutes. After cooling the desired percentages of

buffering materials were added and then 2 per cent inoculum was added. The flasks were then thoroughly shaken and samples removed for determination of original acidity, pH value and counts. All flasks were then incubated at 37°C. and removed only for procuring of later samples.

A 10 c.c. sample was used for acidity titration. After thoroughly shaking the flask the tip of a 10 c.c. pipette was slightly submerged and a sample drawn just up to the zero mark. In this way very little of the culture remained on the outside and on the inside above the mark. It was necessary to rinse the sample from the inside of the pipette. The most convenient method to do this was to use the pipette as a stirring rod. After dilution of the sample with alkali to near the end point the flow was stopped and sample was drawn up into the pipette, allowed to flow out and titrated to the end point.

The hydrogen ion concentration was determined by means of the quinhydrone electrode and saturated calomel half cell. Samples which were coagulated were diluted slightly with distilled water and thoroughly shaken before making the determination.

In preliminary work of this investigation it was found that over 2 per cent of the phosphate salt caused coagulation of the milk, thus it was not satisfactory to use higher per centages.

TABLE V - A

Effect of Calcium Carbonate and Phosphate Buffer on the Growth of Lactobacillus Acidophilus - (Culture 42)

(Counts Reported as Millions)

	Control			$CaCO_3$ 5 per cent			$CaCO_3$ 10 per cent			Buffer 1 per cent			Buffer 2 per cent		
Time Hours	pH	Acid	Count	pH	Acid	Count	pH	Acid	Count	pH	Acid	Count	pH	Acid	Count
0	6.4	.15	7	6.4	.14	7	6.4	.14	6	6.4	.30	7	6.4	.41	7
12	5.9	.40		5.84	.29		5.9	.31		6.0	.72		6.2	.90	
24	5.38	.82	770	5.48	.57	1,100	5.42	.55	1,120	5.7	.72	374	5.9	.82	575
36	5.05	.89	1,290	5.2	.69	1,660	5.32	.77	1,520	5.1	1.43	1,850	5.3	1.55	1,210
48	4.90	1.19	1,430	5.12	.72	1,880	5.24	.72	1,610	4.90	1.93	915	5.1	2.72	1,160
I 60	4.74	1.46	1,250	5.2	.74	1,380	5.20	.76	710	4.74	1.89	1,240	4.85	2.52	600
72	4.61	1.58	940	5.1	.76	590	5.20	1.00		4.63	1.85	289	4.60	2.52	270
84			660			550			495			256			193
96	4.50	1.65	480	4.95	.84	460	5.10	.84	336	4.63	2.10	128	4.48	2.57	86
108	4.38	1.68	510	4.83	1.01	455	4.98	.84	272	4.60	2.14		4.34	2.86	136
120	4.20	1.74	309	4.75		421	4.95	.76	210						
0	6.4	.10	6	6.4	.09	6	6.4	.09	6	6.4	.22	6	6.4	.27	6
12	6.2	.13	128	6.0	.13	135	6.0	.13	224	6.2	.29	49	6.23	.30	53
24	5.55	.168	270	5.55	.24	306	5.55	.16	795	6.12	.24	470	6.15	.35	560
36	5.20	.42	620	5.15	.44	777	5.18	.26	1,210	5.54	.28	660	5.46	.67	840
I 48	5.00	1.16	703	4.66	.94	1,570	5.00	.84	1,650	5.06	1.51	990	4.85	1.56	340
60	4.62	1.36	1,120	4.65	.90	1,740	5.00	.84	1,640	4.85	1.71	1,990	4.63	1.68	134
72	4.48	1.54	752	4.95	.84	844	4.95	.84	1,360	4.63	1.92	603	4.54	1.76	30
84	4.20	1.54	750	4.63	.91	800	4.75	.91	965	4.28	1.94	312	4.30	1.92	4
96	4.10	1.54	535	5.00	1.13	650	5.00	1.08	625	3.92	1.92	369	3.92	1.93	5
108	4.05	1.64	506	4.95	1.16	630	4.95	.91	530	3.92	1.96	320	3.92	1.96	10
120	3.92	1.71	467	4.95	.87	320	4.95	.80	548	3.92	2.06	271	3.92	2.08	5

TABLE V - A (Cont'd)

Effect of Calcium Carbonate and Phosphate Buffer on the Growth of Lactobacillus Acidophilus - Culture 42

(Counts Reported as Millions)

	Control			$CaCO_3$ 10 per cent			Buffer 2 per cent		
Time hours	pH	Acid	Count	pH	Acid	Count	pH	Acid	Count
0	6.2	.17	7	6.2	.17	8	6.2	.57	9
12	6.15								
24	5.5	.45	150	5.4	.52	454	5.8	.80	153
36	5.04	.92	890	5.22	.84	1,600	5.02	1.46	280
48	4.85	1.26	1,110	5.12	.92	1,860	4.7	1.68	395
60	4.75	1.34	1,330	4.85	.84	1,830	4.63	1.82	430
72	4.63	1.54	795	4.70	.77	910	4.55	2.03	155
84	4.5		560	4.82	.87	410	4.44		138
96	4.4	1.68	402	4.95	.87	338	4.03	2.01	140
108	4.4								
120	4.4	1.69	372	4.95	.87	38	4.3	2.15	11
132	4.4		338			24	4.3		9

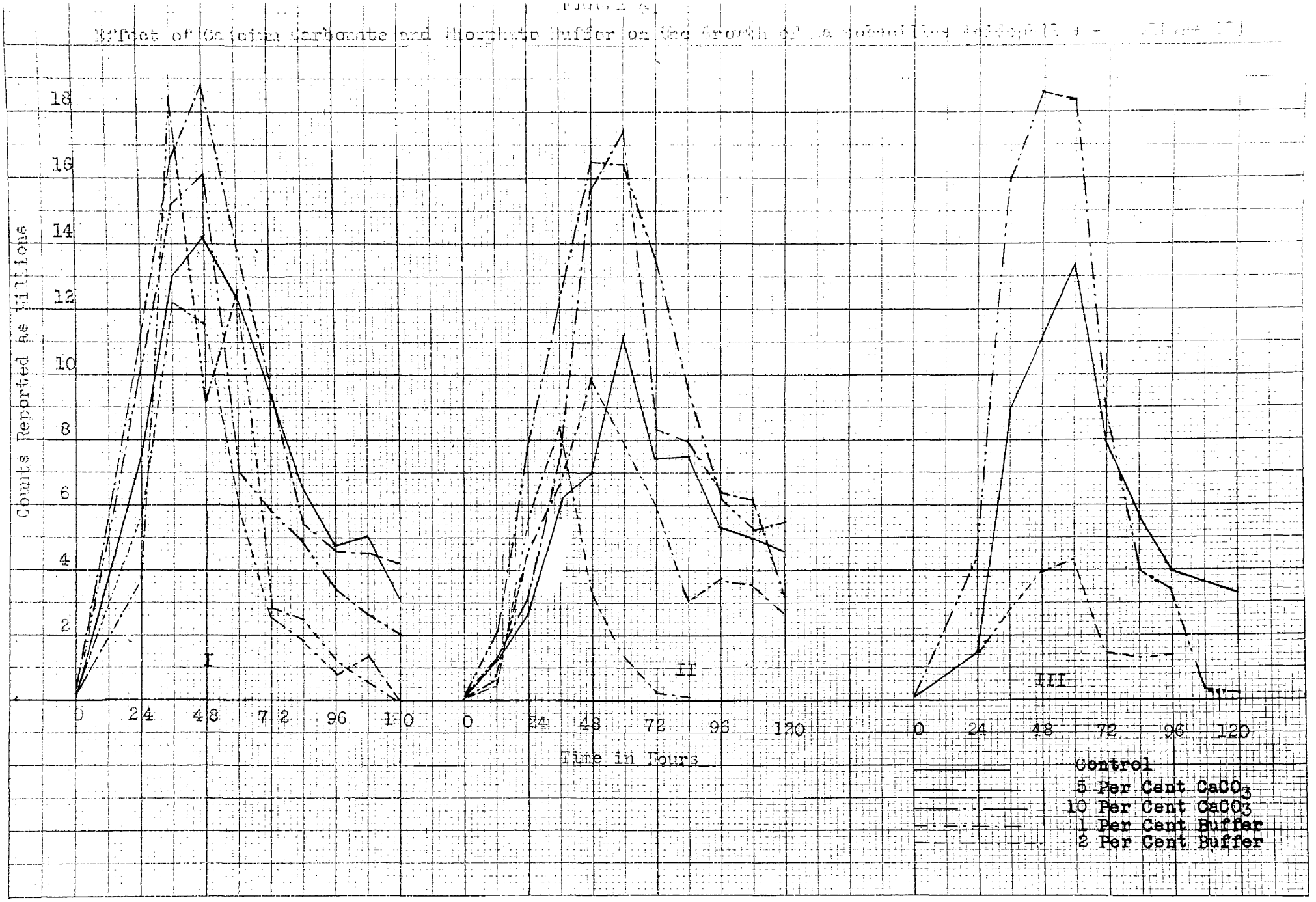

TABLE V - B

Effect of Calcium Carbonate and Phosphate Buffer on the Growth of Lactobacillus Acidophilus - (Culture R5)

(Counts Reported as Millions)

		Control			$CaCO_3$ 5 per cent			$CaCO_3$ 10 per cent			Buffer 1 per cent			Buffer 2 per cent		
	Time Hours	pH	Acid	Count	pH	Acid	Count	pH	Acid	Count	pH	Acid	Count	pH	Acid	Count
	0	6.4	.15	4	6.4	.15	3	6.4	.14	4	6.4	.36	31	6.4	.49	4
	12	5.86	.36	85	6.05	.36	42	5.72	.34	76	5.9	.68	31	5.94	.90	44
	24	5.54	.55	249	5.64	.58	215	6.10	.60	451	5.7	.74	95	5.70	1.05	66
	36	4.75	1.16	455	5.44	.67	875	5.32	.69	1,330	5.4	.97	109	5.32	1.21	51
	48	4.35	1.42	555	5.32	.72	1,100	5.30	.69	1,400	5.1	1.36	85	5.20	1.33	30
	60	4.10	1.51	480	5.22	.77	720	5.46	.81	885	5.0	1.61	13.5	5.15		37
	72	3.95	1.59	334	5.10	.80	493	5.30	.71	495	4.0	1.74	8.5	5.05	1.48	9
I	84	3.90	1.68	290	4.95	.68	338	5.30		243			6.5			0
	96	3.98	1.74	183	4.82	.67	392	5.30	.57	258	4.72	1.84	8.5	5.00	1.63	0
	108	3.80	1.82	112	5.00	.69	179	5.20	.59	195	4.30	1.90	10	4.85	1.65	0
	120	3.74	1.96	37	4.95	.67	74	5.05	.60	76	4.20	1.96	2	4.65	1.74	0
	132	3.76	2.04	15	4.90	.71	20	5.00	.71	44	4.15	1.96	3.5	4.60	1.76	0
	0	6.0	.24	3	6.2	.10	3	6.2	.10	3	6.2	.65	2.9	6.2	.86	3
	12	5.7	.38	75	5.95	.30	69	6.05	.27	52	5.8	.84	51	5.95	.95	52
	24	5.52	.72	21	5.3	.63	298	5.3	.49	311	5.2	1.09	0	5.2	1.41	78
	36	4.72	1.12	485	4.95	.88	1,110	5.14	.68	780	4.91	1.58	1	4.82	1.84	70
	48	4.20	1.61	500	4.80	.98	1,260	5.00	.69	975	4.72	1.79	4	4.65	2.21	33
II	60	4.15	1.81	420	4.70	.91	483	4.90	.73	520	4.60	2.09	2	4.60	2.45	20
	72	4.15	1.82	307	4.80	1.07	422	4.85	.76	295	4.45	2.14	0	4.36	2.75	16
	84	4.20	1.93	200	4.85	.79	302	4.85	.76	237	4.45	2.16	0	4.10	2.81	25
	96	4.20	2.04	7	4.68	.89	32	4.85	.76	8	4.20	2.29	2.5	4.10	2.88	13
	108	4.15	2.08	7	4.85	.92	9	4.88	.76	5	4.12	2.31	0	4.10	2.88	2

TABLE V - B (Cont'd)

Effect of Calcium Carbonate and Phosphate Buffer on the Growth of Lactobacillus Acidophilus - (Culture R5)

(Counts Reported as Millions)

Time Hours	Control			$CaCO_3$ 10 per cent			Buffer 2 per cent		
	pH	Acid	Count	pH	Acid	Count	pH	Acid	Count
0	6.2	.194	139	6.2	.184	14	6.2	.184	15
12									
24	5.52	.789	446	5.04	.599	737	5.51	1.008	88
36	4.8	1.22	735	5.00	.688	1,050	5.12	1.69	120
48	4.46	1.44	605	4.95	.672	1,110	4.60	1.84	25
60	4.28	1.46	550	4.90	.688	540	4.54		25
72	4.12	1.59	345	4.8	.605	279	4.30	2.01	4
84	4.05	1.68	309	4.82	.599	303	4.32		0
96	3.95	1.71	258	4.95	.604	137	4.3	2.10	0
108	3.88	1.74	183	5.00	.55	69	4.24		0
120	3.85	1.80	136	5.12	.55	14	4.15	2.68	0
132	3.72	1.92	20	5.00		4	4.10		0

FIGURE B.

Effect of Calcium Carbonate and Phosphate Buffer on the Growth of Lactobacillus Acidophilus - Culture 25

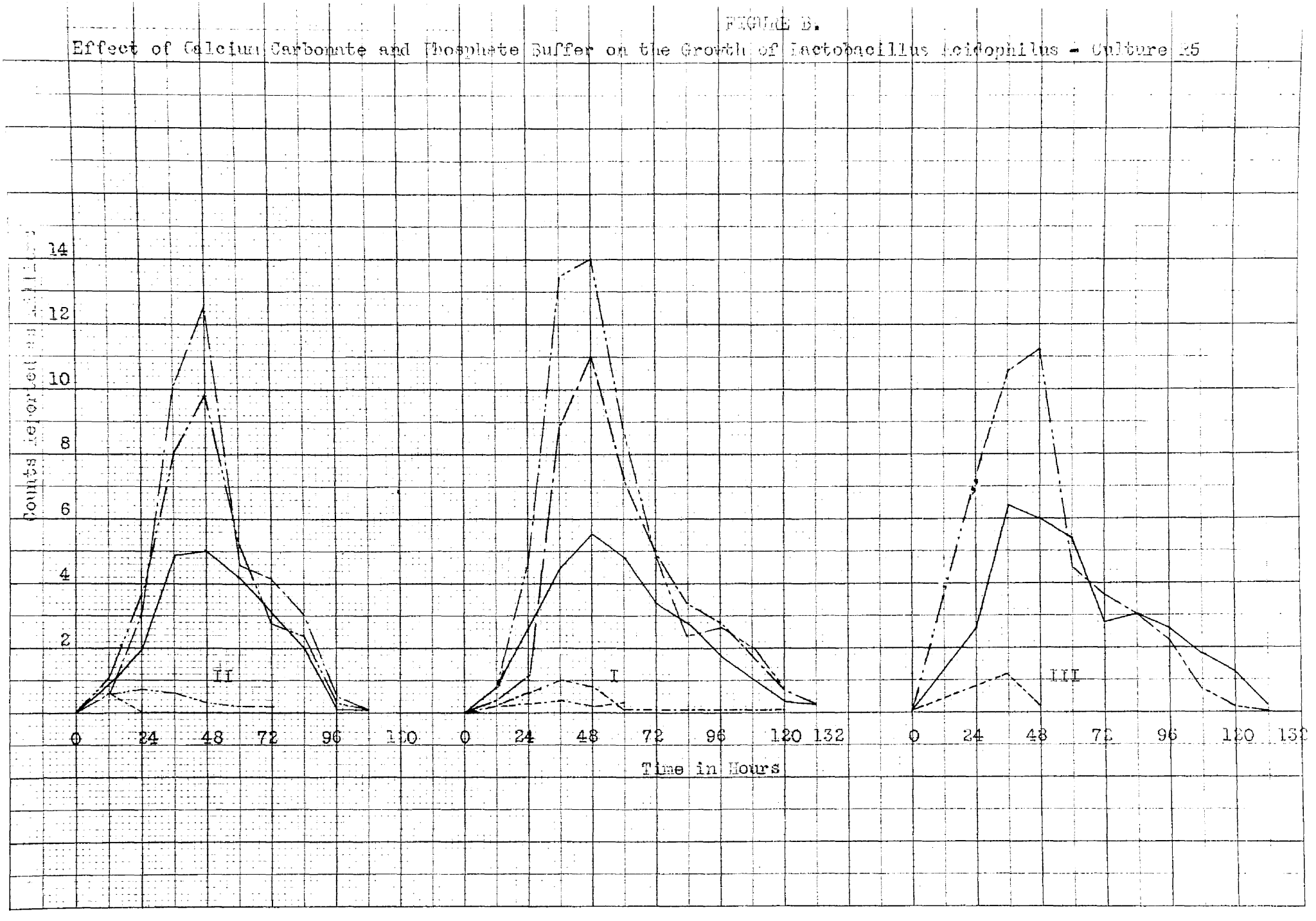

TABLE V - C

Effect of Calcium Carbonate and Phosphate Buffer on the Growth of Lactobacillus Acidophilus - (Culture H)

(Counts Reported as Millions)

Time Hours	Control			Buffer 1 per cent			Buffer 2 per cent			$CaCO_3$ 5 per cent			$CaCO_3$ 10 per cent		
	pH	Acid	Count	pH	Acid	Count	pH	Acid	Count	pH	Acid	Count	pH	Acid	Count
0	6.4	.10	7	6.4	.22	9	6.4	.27	8	6.4	.09	6.6	6.4	.09	7
12	6.05	.11	18	6.2	.20	18	6.22	.29	17	6.05	.11	15	6.1	.09	15
24	5.75	.14	403	6.04	.27	298	6.15	.30	183	5.70	.15	510	5.70	.15	506
36	4.95	.26	387	5.60	.46	406	5.52	.42	70	4.90	.55	792	5.42	.30	970
48	4.63	.91	428	5.10	1.26	211	4.81	1.54	168	4.45	.66	682	5.05	.92	653
60	4.44	1.09	202	4.78	1.46	171	4.71	1.68	148	4.46	.72	454	4.82	1.09	632
72	4.32	1.23	119	4.41	1.61	150	4.63	1.85	123	4.82	.74	365	4.90	1.05	550
84	4.18	1.26	67	4.35	1.68	134	4.42	1.91	195	4.80	.96	395	4.72	1.09	557
96	3.95	1.26	119	4.15	1.82	104	4.25	1.80	123	5.05	.81	182	4.68	.77	311
108	3.95	1.26	78	4.08	1.82	132	4.25	1.85	191		.91	458	4.65	.81	505
120	3.92	1.31	69	4.00	1.85	79	4.40	1.85	148	4.80	.84	338	4.63	.92	257

TABLE V - C (Cont'd)

Effect of Calcium Carbonate and Phosphate Buffer on the Growth of Lactobacillus Acidophilus - Culture H

(Counts Reported as Millions)

	Time hours	Control pH	Control Acid	Control Count	$CaCO_3$ 10 per cent pH	$CaCO_3$ 10 per cent Acid	$CaCO_3$ 10 per cent Count	Buffer 2 per cent pH	Buffer 2 per cent Acid	Buffer 2 per cent Count
	0	6.2	.19	12	6.2	.18	13	6.2	.57	11
	12	5.85	.28	34	6.05	.24	40	6.10	.76	44
	24	5.0	.51	281	5.0	.53	274	5.8	.88	163
	36	4.92	.92	405	4.63	.76	755	5.0	1.46	210
	48	4.00	1.12	370	4.95	.90	700	4.68	1.63	75
II.	60			385	4.88	.90	385	4.55	1.76	24
	72	4.20	1.34	272	4.88	.96	238	4.32	1.97	53
	84			350			239			130
	96	4.05	1.46	106	4.63	.71	40	4.35	2.10	11
	108									11
	120	3.80	1.72	101	4.95	.84		4.13	2.25	7
	132			104		.96	14			7
	0	6.2	.28	10	6.2	.25	12	6.2	.47	9
	12									
	24	5.12	.45	279	5.33	.46	250	5.84	1.05	148
	36									
	48	4.36	1.18	515	4.75	.74	775	4.80	2.09	133
II.	60			385			500	4.05	2.14	295
	72	4.25	1.34	310	4.72		425	4.52	2.19	175
	84			329			300			126
	96	4.00	1.60	197	4.77		152	4.25	2.62	114
	108									
	120	3.88	1.78	114	4.90		53	4.10	2.74	73

Effect of Calcium Carbonate and [illegible] on the Growth of [illegible] Acido[illegible]

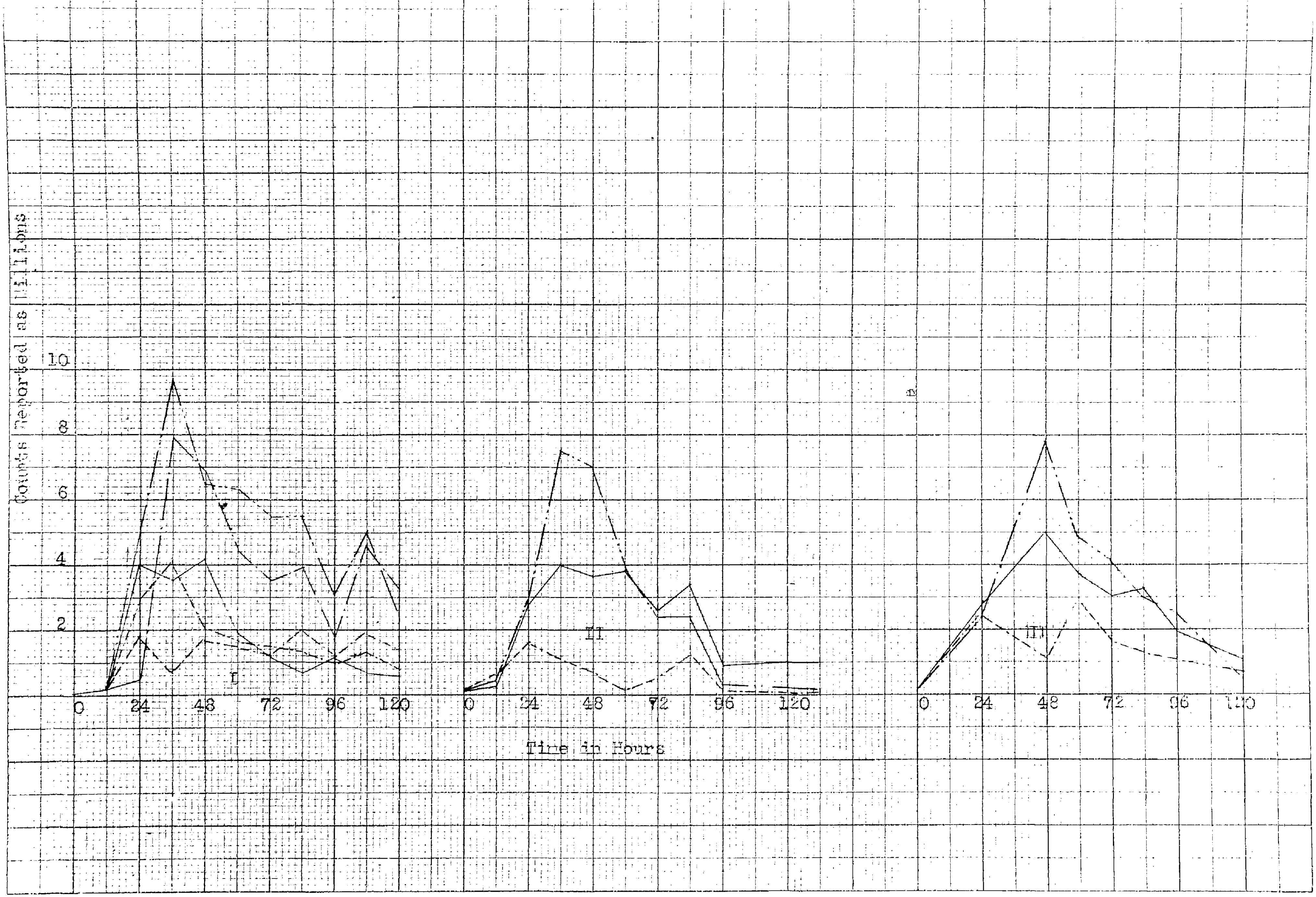

TABLE V - D

Effect of Calcium Carbonate and Phosphate Buffer on the Growth of Lactobacillus Acidophilus - Culture R_2

(Counts Reported as Millions)

Time hours	Control			$CaCO_3$ 10 per cent			Buffer 2 per cent		
	pH	Acid	Count	pH	Acid	Count	pH	Acid	Count
0	6.2	.28	7	6.2	.25	8	6.2	.47	8
12									
24	4.82	.76	266	5.0	.68	401	5.2	1.10	129
36									
48	4.32	1.35	510	4.9	.87	535	4.48	1.94	141
60			450			525			83
72	4.10	2.04	450	4.8	.74	315	4.42	2.62	163
84			285						68
96	3.9	2.46	193	4.95	.72	7	4.38	3.20	46
108									
120	3.75	2.50	33	5.00	.63	9	4.00	3.31	14

TABLE V - E

Effect of Calcium Carbonate and Phosphate Buffer on the Growth of Lactobacillus Acidophilus - (Culture I-6)

(Counts Reported as Millions)

	Control			$CaCO_3$ 10 per cent			Buffer 2 per cent		
Time Hours	pH	Acid	Count	pH	Acid	Count	pH	Acid	Count
0	6.2	.28	11	6.2	.25	10	6.2	.47	8
12	5.85	.63	347	5.95	.40	465	5.75	.90	154
24	4.8	.90	720	5.04	.89	1,190	5.12	1.58	12
36	4.42	1.55	775	5.00	.80	1,360	4.98	1.76	35
48	4.28	1.48	820	4.80	.85	845	4.80	2.15	13
60	4.15	1.70	480	4.70	.80	975	4.80	2.15	11
72	4.12	1.64	390	4.92	.82	740	4.72	2.17	8
84			345			655			6
96	3.87	1.75	179	5.00	.98	910	4.46	2.44	1
108									
120	3.80	1.81	19	5.00	.79	715	4.30	2.52	0
0	6.2	.15	11	6.2	.101	10	6.2	.25	12
12	5.85	.40	327	5.85	.30	396	5.63	.82	172
24	5.05	.81	620	5.2	.63	1,210	5.24	1.55	200
36	4.60	1.19	710	5.05	.82	1,480	4.85	1.89	132
48	4.42	1.30	910	4.95	.82	900	4.63	1.83	73
60	4.30	1.58	475	5.00	.91	1,170	4.55	2.15	17
72	4.20	1.61	385	4.85	.85	835	4.44	2.26	28
84	4.05	1.74	365	4.85	.90	626	4.63	2.17	
96	3.95	1.86	259	5.10	.91	901	4.58	2.21	7
108	3.95	1.91	193	5.04	.91	712	4.48	2.33	8
120	4.00	1.98	179	5.04	.98	741	4.32	2.44	5
132	3.98	1.98	75	5.15	.80	580	4.28	2.52	3

FIGURE E.

Effect of Calcium Carbonate and Phosphate Buffer on the Growth of Lactobacillus Acidophilus - Culture L-6

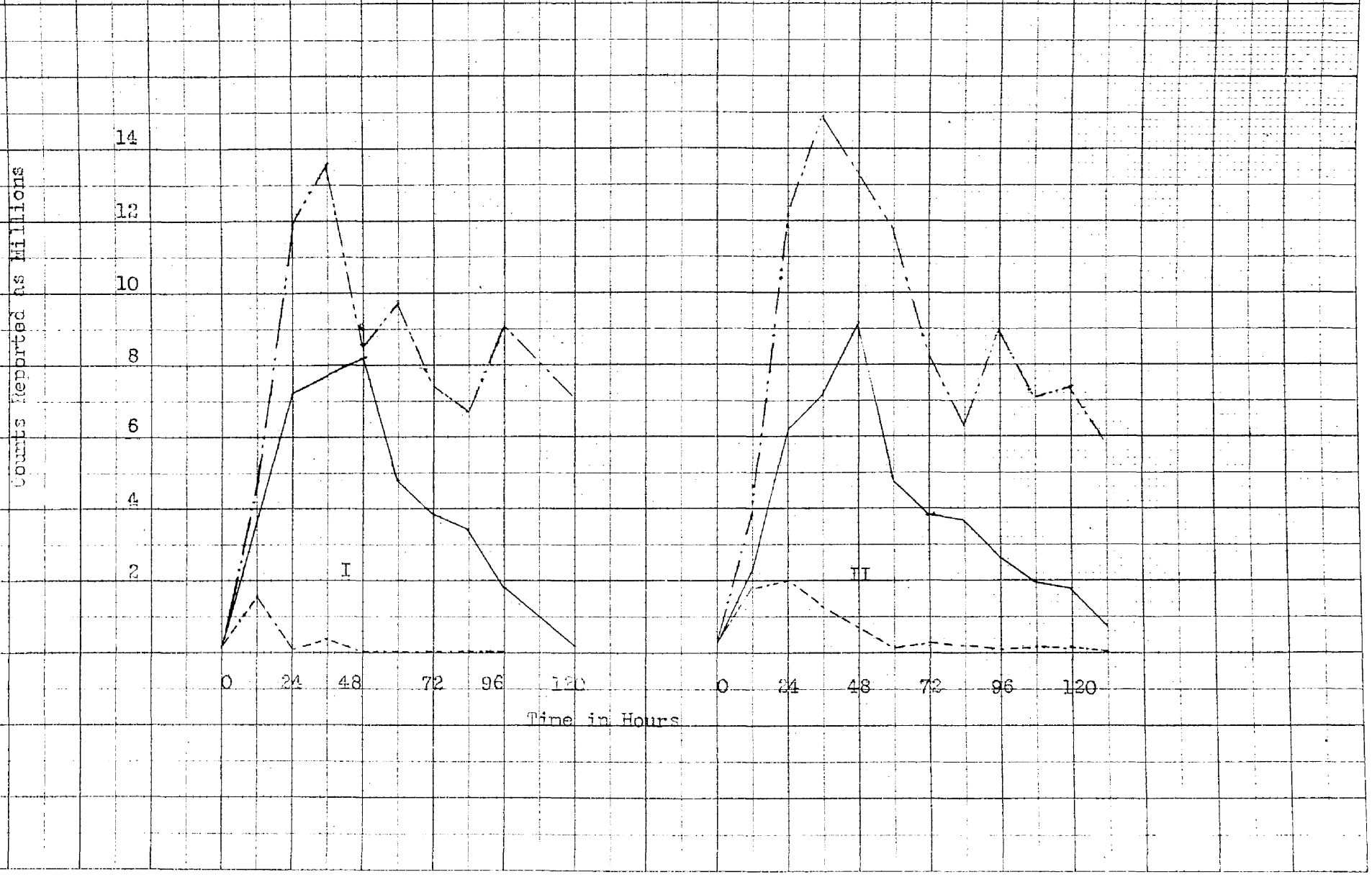

TABLE V - F-1

Effect of Calcium Carbonate on the Growth of Lactobacillus Acidophilus - (Culture K)

(Counts Reported as Millions)

	Control			$CaCO_3$ 5 per cent			$CaCO_3$ 10 per cent		
Time Hours	pH	Acid	Count	pH	Acid	Count	pH	Acid	Count
0	6.3	.36	20	6.3	.28	17	6.4	.27	19
12	5.72	.47	119	5.8	.50	178	5.8	.44	214
24	4.80	1.01	749	4.62	1.01	2,000	4.72	.92	2,000
36	4.18	1.74	2,900	4.90	1.76	2,500	5.1	1.43	4,900
48	4.00	1.81	3,190	4.82	1.30	4,500	5.05	1.21	3,845
60	3.9	2.12	3,740	4.72	1.58	3,000	4.95	1.23	4,620
72	3.85	2.18	2,790	4.75	1.64	7,270	5.3	1.25	7,885
84	3.75	2.37	3,470	4.75	1.85	5,360	6.3	1.43	5,220
96	3.75	2.70	2,700	4.75	2.11	5,920	6.2	1.24	3,470
108	3.75	2.72	2,800	4.72	1.80	3,800	5.3	.83	1,480
120	3.60	2.75	2,520	4.60	2.01	3,990		1.04	1,500
0	6.2	.23	25	6.2	.23	26	6.2	.22	21
12	5.62	.44	120	5.8	.32	113	5.8	.32	106
24	4.63	.82	850	5.05	.64	982	4.92	.77	665
36	4.15	1.58	2,220	4.94	1.04	2,570	5.05	1.19	2,245
48	4.1	1.67	2,950	5.05	1.18	3,320	5.05	1.01	4,980
60	3.8	2.08	3,110	5.05	1.24	4,710	5.05	1.05	6,580
72	3.75	2.08	2,870	4.63	1.37	3,040	4.67	1.19	5,795
84	3.55	2.36	2,890	4.45	1.37	5,070	4.63	.95	2,625
96	3.47	2.46	1,680	4.73	1.26	6,110	5.02	.86	355
108	3.42	2.54	1,000	4.85	1.02	960	5.38	.67	165
120	3.4	2.66	135	5.08	1.02	405	5.42	.82	305

FIGURE F-1

Effect of Calcium Carbonate on the Growth of Lactobacillus Acidophilus - (Culture H)

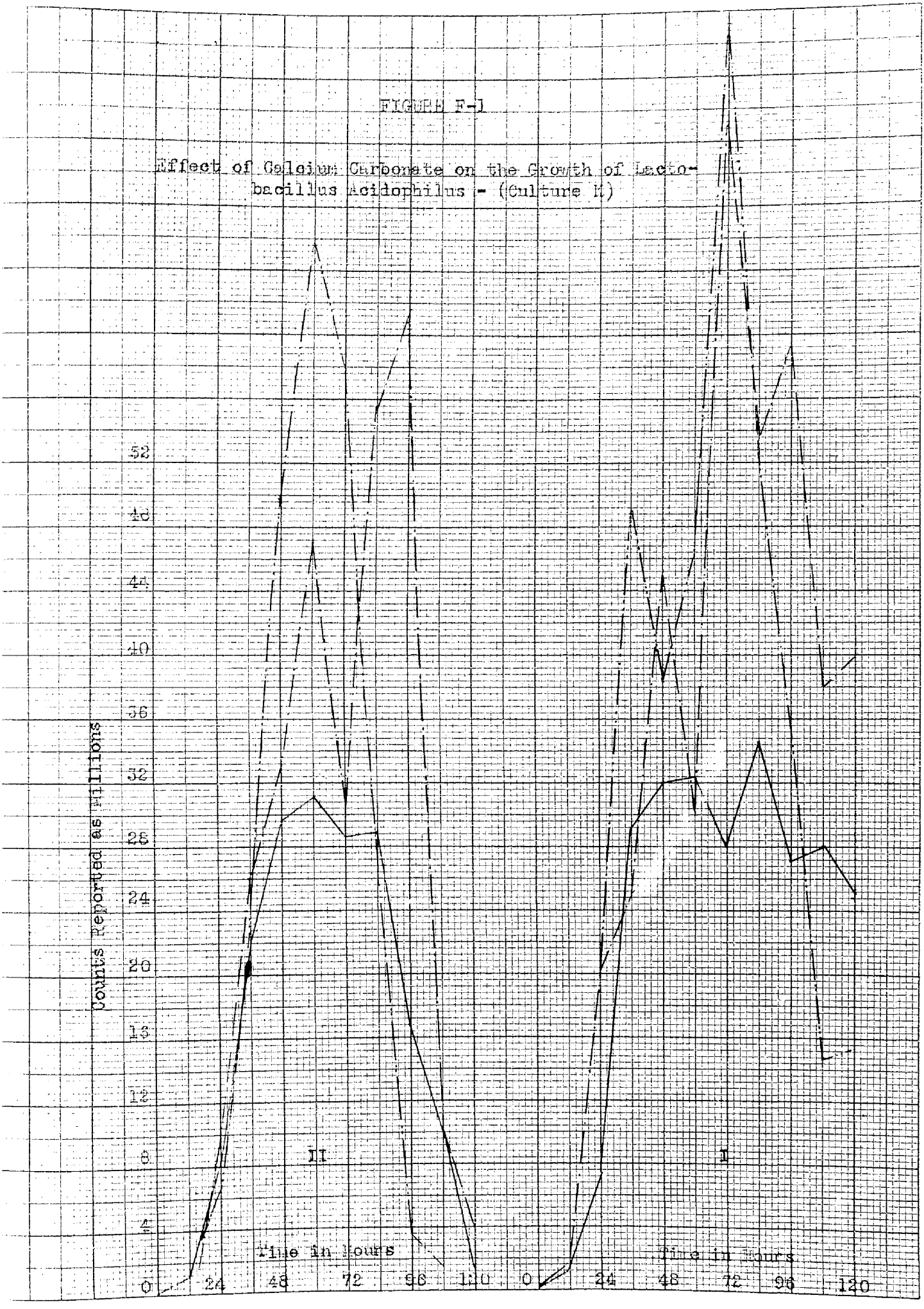

TABLE V - F-2

Effect of Calcium Carbonate and Phosphate Buffer on the Growth of Lactobacillus Acidophilus - (Culture K)

(Counts Reported as Millions)

		Control			Buffer 1 per cent			Buffer 2 per cent		
	Time Hours	pH	Acid	Count	pH	Acid	Count	pH	Acid	Count
	0	6.2	.24	13	6.2	.58	15	6.2	.05	15
	12	5.55	.45	238	5.84	.68	156	6.03	1.03	218
	24	4.81	.59	345	5.00	.97	228	5.39	1.23	186
	36	4.00	1.12	1,970	4.35	1.51	1,550	4.36	1.93	1,310
	48	3.85	1.37	2,150	3.96	1.80	1,830	3,96	2.27	2,020
I.	60	3.71	1.58	1,820	4.22	1.84	2,470	3.84	2.63	2,440
	72	3.60	1.67	1,100	4.15	2.93	2,610	3.80	2.72	2,290
	84	3,72	1.98	677	3.78	3.28	2,880	3.82	2.81	2,600
	96	3.67	1.73	318	3.89	3.48	3,200	3,79	2.90	2,460
	108	3.68	1.94	192	3.80	3.69	2,820	3.65	3.04	1,670
	120	3.54	1.60	184	3.60	3.71	1,510	3.55	3.10	1,400
	132	3.65	1.61	127	3.58	4.01	1,780	3.54	3.14	635
	0	6.3	.27	25	6.65	.45	17	6.7	.63	12
	12	5.8	.31	148	6.35	.54	156	6.55	.69	131
	24	5.3	.61	505	6.00	.74	635	6.4	.86	375
	36	4.6	1.17	2,160	5.0	1.28	1,320	5.55	1.35	710
	48	4.42	1.53	2,580	4.5	2.01	2,035	4.7	2.17	1,490
II.	60	4.2	1.60	2,270	4.3	2.16	3,090	4.32	2.67	2,535
	72	4.1	1.85	contam.	4.1	2.30	1,950	4.21	2.81	2,935
	84	3.9	1.86	1,560	3.95	2.33	1,230	4.12	2.91	2,695
	96	3.7	1.98	660	3.82	2.52	685	3.80	3.60	1,960
	108	3.6	2.20	830	3.78	2.97	615	3.80	3.33	1,630

FIGURE P-2

Effect of Phosphate Buffer on the Growth of Lactobacillus Acidophilus - (Culture K)

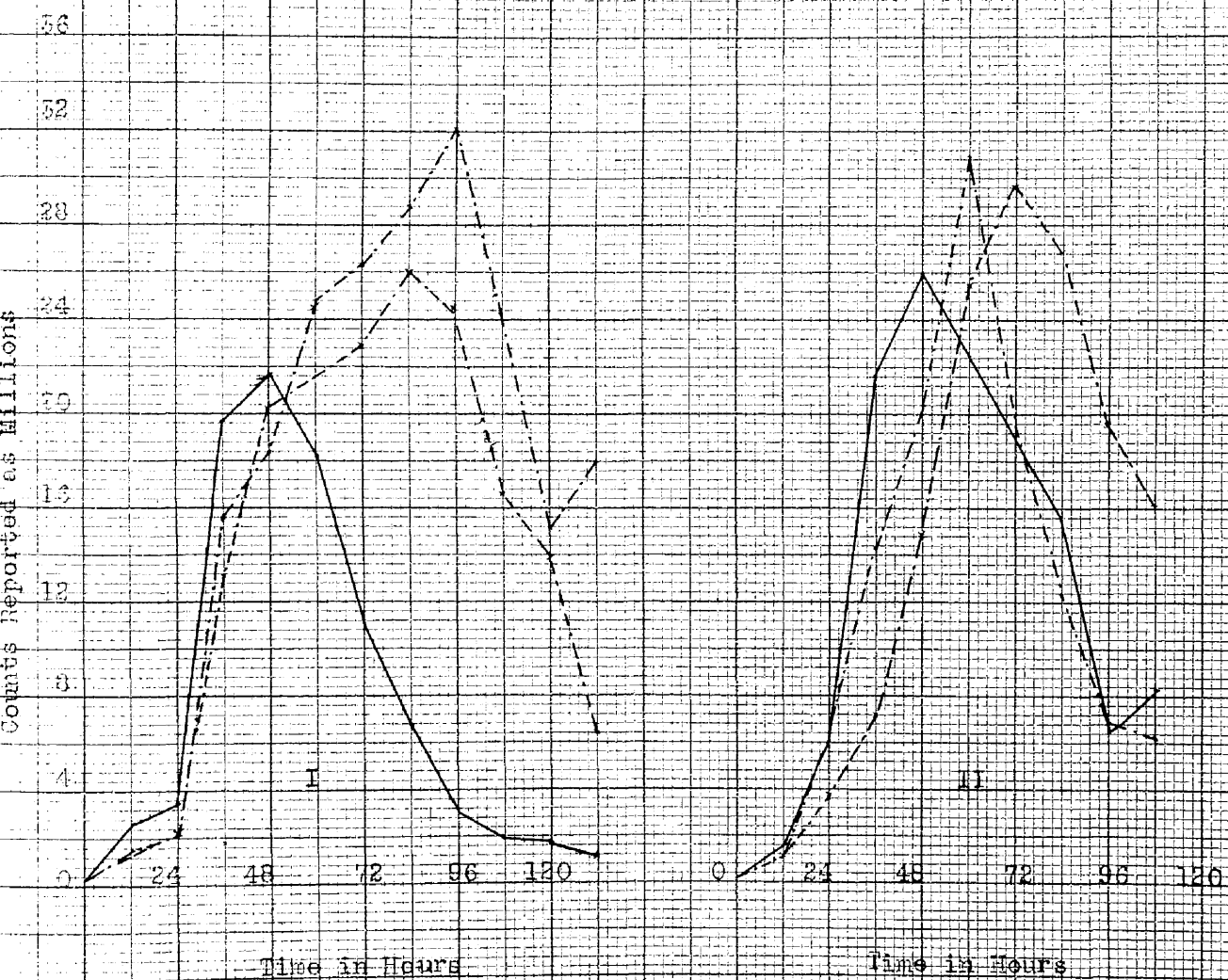

TABLE V - G

Effect of Calcium Carbonate and Phosphate Buffer on the Growth of Lactobacillus Acidophilus - Culture K4Y

(Counts Reported as Millions)

Time hours	Control			$CaCO_3$ 10 per cent			Buffer 2 per cent		
	pH	Acid	Count	pH	Acid	Count	pH	Acid	Count
0	6.2	.17	17	6.2	.17	21	6.2	.57	19
12	5.55	.29	465	5.85	.53	735	6.1	.67	800
24	4.95	.51	1,110	5.02	.61	1,700	5.72	.81	1,495
36	5.00	.87	1,620	4.50	.67	2,770	5.00	1.42	1,225
48	4.68	.95	2,120	5.30	.95	3,540	4.63	1.76	2,050
60	4.25	1.26	2,460	5.05	.90	5,750	4.44	1.83	2,630
72	3.92	1.33	2,290	4.82	.76	5,950	4.18	2.10	2,420
84			1,720	4.85	.76	7,500			1,900
96	4.90	1.42	1,100	4.95	.77	2,360	4.30	2.26	1,570
108									
120	3.88	1.72	700	5.00	.71	1,280	4.02	2.92	1,370
132	3.70	1.92	528	5.05	.71	960	4.05	3.04	1,090
0	6.2	.28	21	6.2	.25	19	6.2	.47	22
12	5.85	.29	400	5.95	.28	510	6.02	.62	505
24	5.14	.53	820	5.2	.65	1,970	5.63	1.17	1,090
36	4.63	.74	865	4.95	.91	3,310	5.2	1.26	1,755
48	4.30	1.12	1,260	4.63	1.03	5,710	4.45	1.43	1,655
60	4.28	1.26	1,600	4.72	.81	6,880	4.24	2.24	2,090
72	4.05	1.49	1,520	4.63	.75	6,070	4.15	2.73	1,355
84			1,170			5,570			1,290
96	3.9	1.75	1,100	5.00	.74	3,370	3.98	2.96	1,005
108									
120	3.82	1.92	1,490	5.02	.64	1,850	3.96	3.06	2,345

FIGURE G.

Effect of Calcium Carbonate and Phosphate Buffer on the Growth of Lactobacillus Acidophilus -(Culture K4y)

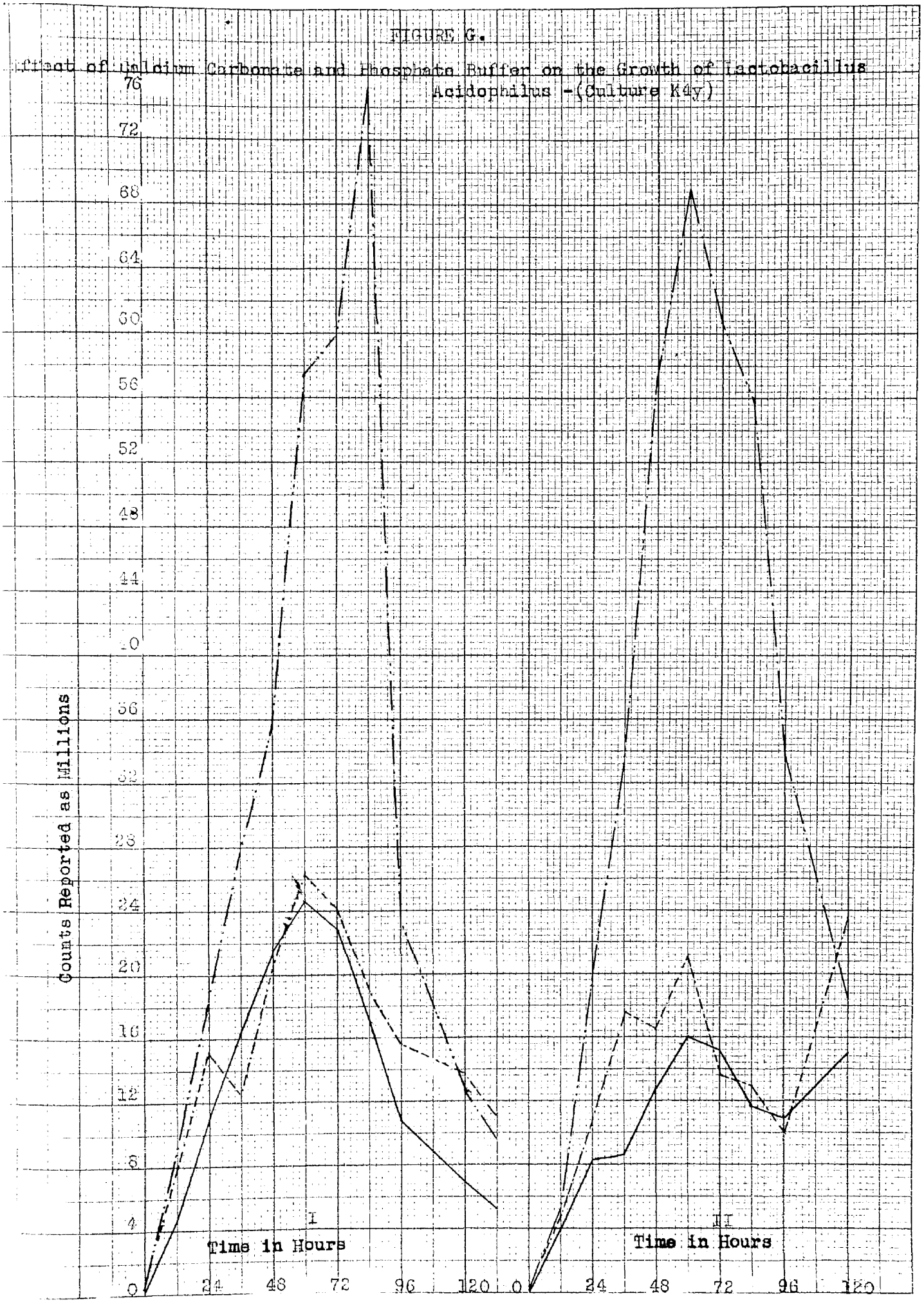

TABLE V - H

Effect of Calcium Carbonate and Phosphate Buffer on the Growth of Lactobacillus Acidophilus - (Culture 49)

(Counts Reported as Millions)

		Control			$CaCO_3$ 5 per cent			$CaCO_3$ 10 per cent			Buffer 1 per cent			Buffer 2 per cent		
	Time Hours	pH	Acid	Count	pH	Acid	Count	pH	Acid	Count	pH	Acid	Count	pH	Acid	Count
	0	6.2	.29	9	6.2	.35	64	6.2	.28	7	6.2	.58	10	6.2	.68	8
	12	5.1	.41	242	5.6	.36	212	11.8	.34	265	6.05	.72	179.5	6.05	1.01	196
	24	4.9	.63	785	5.24	.56	885	5.24	.54	1,095	5.25	.89	925	5.40	1.23	791
	36	4.3	1.17	1,240	5.3	.81	1,330	5.3	.76	1,200	5.00	1.26	1,820	5.15	1.58	1,700
	48	3.94	1.59	1,860	5.02	1.02	1,880	5.18	.89	2,150	4.68	1.37	2,225	4.85	1.67	2,350
I.	60	3.85	1.70	2,020	5.00	.99	2,440	5.0	.84	1,895	4.40	1.51	2,630	4.05	1.93	2,800
	72	3.75	1.75		4.95	.85	2,060	5.05	.69	840	4.05	2.59	2,150	3.84	2.45	2,330
	84	3.94	1.94	1,550	5.30	.90	1,360	5.32	.72	620	3.85	2.88	1,655	3.72	2.73	1,800
	96	3.80	1.96	1,120	5.23	.79	1,060	5.44	.66	335	3.80	2.89	495	3.60	3.03	697
	108	3.75	2.01	710	5.15	.85	805	5.40	.84	500	3.75	2.88	152	3.64	3.14	481
	120	3.70	2.17	375	5.05	.96	500	5.40	.84	450	3.70	2.88	55	3.60	3.14	194
	0	6.2	.28	13	6.2	.20	11	6.2	.20	13	6.2	.45	11.9	6.2	.84	10
	12	5.85	.42	314	5.65	.26	423		.29	403	5.9	.59	364	6.05	.97	364
	24	4.85	.94	795	5.42	.42	1,050	5.63	.74	875	5.42	1.23	975	5.52	.42	910
	36	4.42	1.07	1,620	5.20	.95	1,870	5.50	.84	1,850	4.85	1.58	1,920	5.05	1.58	1,770
	48	3.95	1.46	870	5.45	.78	2,560	5.05	1.16	2,670	4.55	1.71	2,095	4.63	1.93	2,400
II.	60	3.84	1.85	1,550	5.40	.94	2,260	5.05	.95	2,275	3.95	2.25	2,310	4.42	2.33	2,560
	72	3.88	1.91	1,160	5.00	1.07	1,940	5.10	.96	2,000	3.85	2.60	1,885	3.85	2.88	4,000
	84	3.80	2.08	890	4.95	1.18	1,550	4.95	.86	1,770	3.72	2.81	1,110	3.65	3.21	1,550
	96	3.70	2.08	840	4.90	.95	1,200	4.85	.95	1,540	3.65	2.78	610	3.62	3.42	587
	108	3.65	2.16	265	4.86	.93	850	4.85	.96	875	3.60	2.78	405	3.55	3.60	521
	120	3.60	2.26	180	4.95	1.04	690	4.90	.81	830	3.60	2.92	217	3.55	3.63	136
	132	3.55	2.36	123	5.05	.80	390	5.12	1.01	458	3.60	2.97	76	3.50	3.42	35

FIGURE H.

Effect of Calcium Carbonate and Phosphate Buffer on the Growth of Lactobacillus Acidophilus - Culture 49

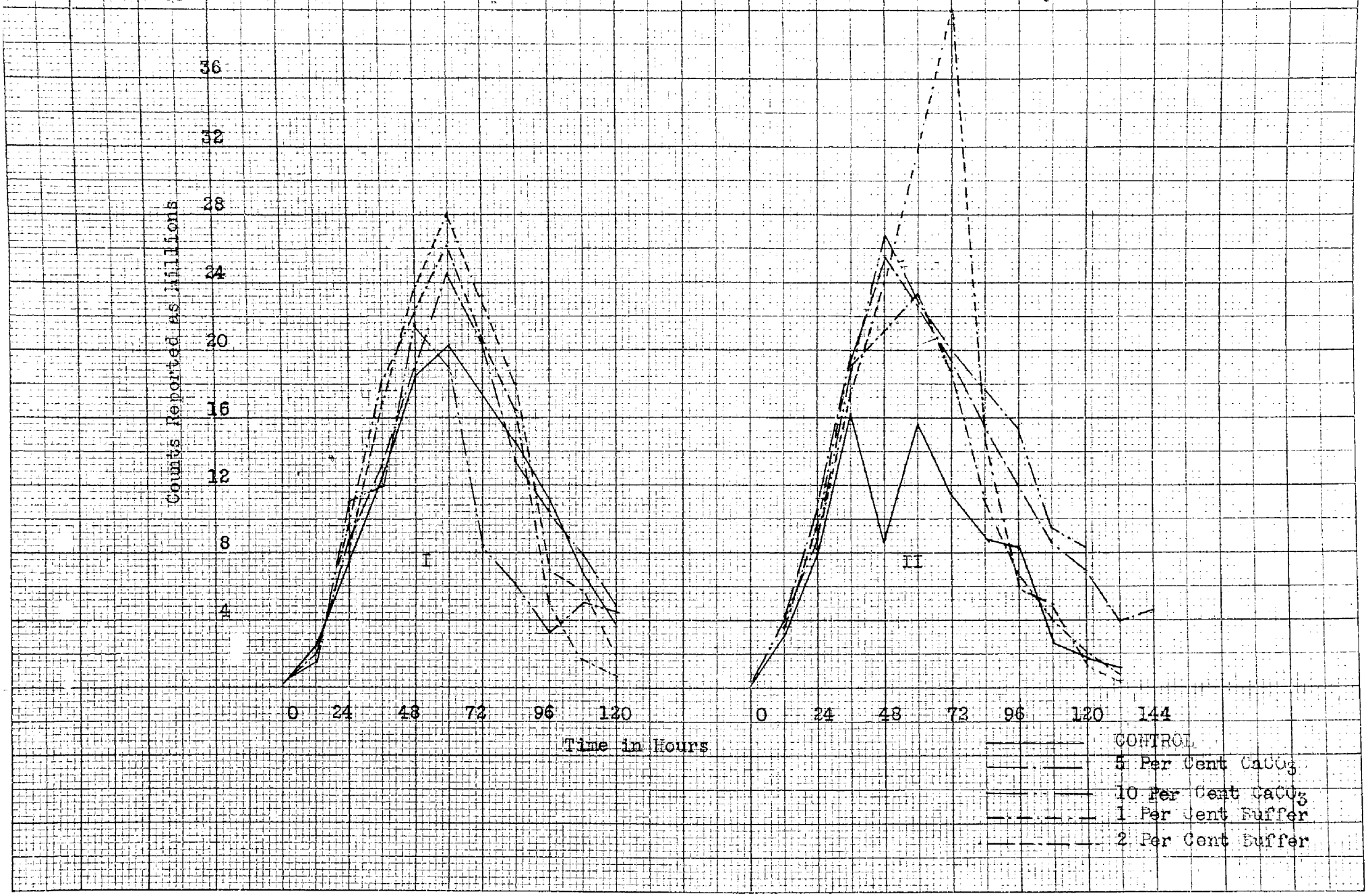

TABLE V - I

Effect of Calcium Carbonate and Phosphate Buffer on the Growth of Lactobacillus Acidophilus - (Culture 73)

(Counts Reported as Millions)

	Control			$CaCO_3$ 5 per cent			$CaCO_3$ 10 per cent		
Time Hours	pH	Acid	Count	pH	Acid	Count	pH	Acid	Count
0	6.4	.26	11	6.4	.20	19	6.4	.20	18
12	5.82	.42	225	5.4	.40	413	5.45	.36	452
24	4.68	.80	840	5.2	.74	1,503	5.2	.76	1,500
36	4.25	1.30	2,460	5.0	.77	2,735	4.95	.81	3,475
48	4.1	1.49	2,525	5.3	1.07	4,025	5.3	1.04	4,125
I. 60	4.02	1.74	2,410	5.1	.95	5,350	5.41	.94	2,910
72	3.88	1.76	1,430	4.95	.76	3,640	5.25	.67	420
84	4.04	1.59	464	5.46	.51	380	5.60	.61	40
96	4.02	1.94	113	5.38	.63	222	5.52	.50	17
0	6.4	.29	12	6.4	.20	13	6.4	.20	11
12	5.72	.41	178	5.9	.47	401	5.5	.26	238
24	4.76	.96	795	4.85	.78	690	5.35	.67	820
36	4.3	1.26	1,815	4.82	1.11	2,100	5.2	.81	2,555
48	4.1	1.74	2,185	4.74	1.12	2,500	5.4	.94	3,810
I. 60	4.05	1.85	2,330	4.74	1.11	3,780	5.05	1.07	4,360
72	3.92	1.86	2,090	4.72	1.01	4,890	5.10	1.26	4,020
84	3.88	1.91	1,665	4.95	.95	3,730	4.80	.95	2,555
96	3.85	2.08	710	4.60	.93	1,510	4.95	.81	845
108	3.80	2.08	156	4.80	.95	365	5.10	.78	452.5
120	3.65	2.20	38	4.74	.86	108	5.25	.72	351

FIGURE I.

Effect of Calcium Carbonate and Phosphate Buffer on the Growth of Lactobacillus Acidophilus - (Culture 73)

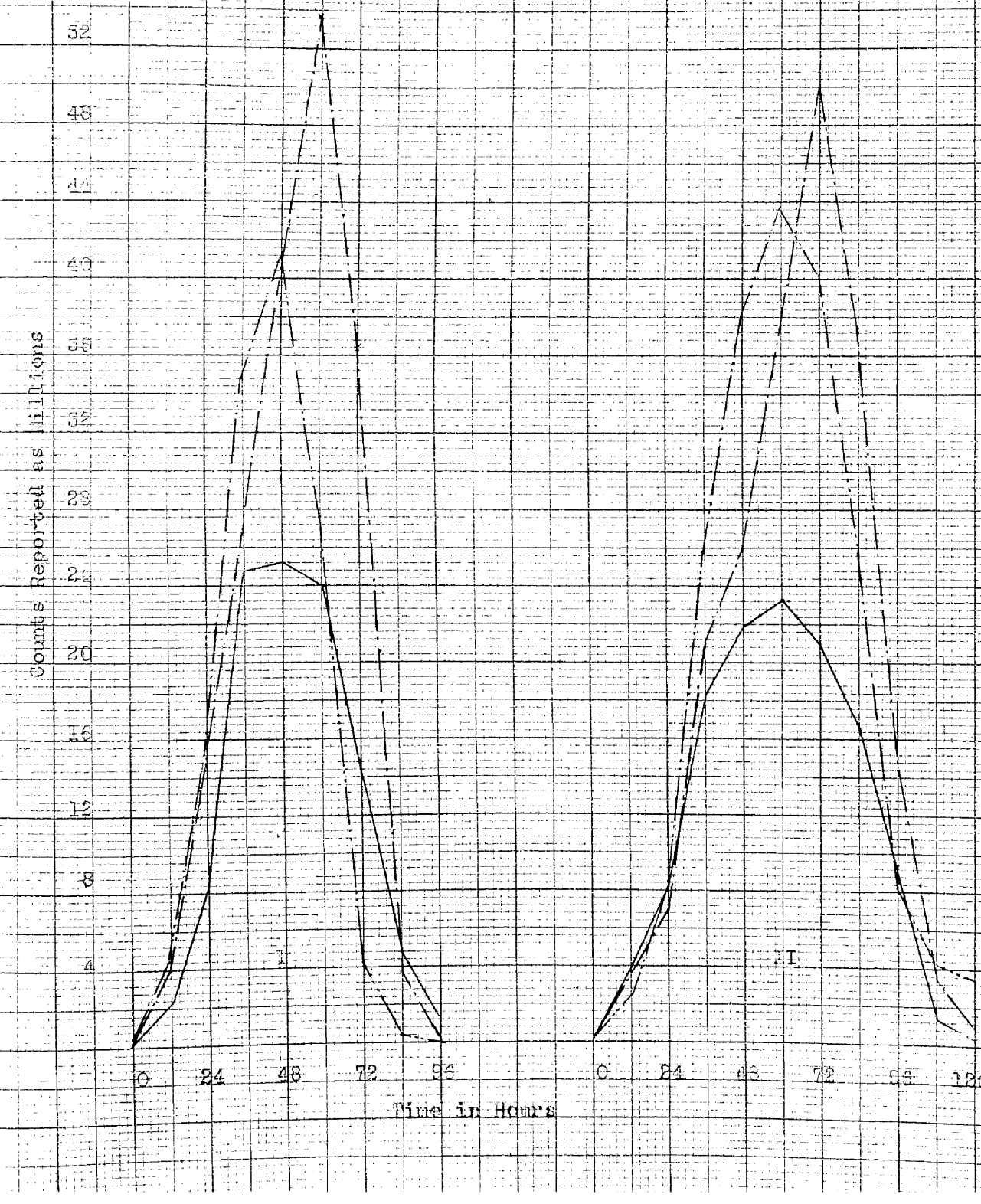

TABLE VI

Averages of Highest Counts, Percentages of Acid, and pH Values of Lactobacillus Acidophilus
(Counts in Millions per c.c.)

Culture	Control			$CaCO_3$ 5 per cent			$CaCO_3$ 10 per cent			Buffer 1 per cent			Buffer 2 per cent		
	pH	Acid	Count	pH	Acid	Count	pH	Acid	Count	pH	Acid	Count	pH	Acid	Count
K	3.78	2.17	3,290	4.6	1.50	6,520	5.17	1.15	7,230						
	4.13	1.45	2,320							4.1	2.8	3,140	4.02	2.81	2,760
49	3.85	1.77	1,780	5.23	.885	2,500	5.05	.895	2,080	4.2	1.88	2,470	3.95	2.40	3,400
K4y	4.26	1.26	2,030				4.79	.785	7,190				4.4	2.03	2,360
73	4.15	1.57	2,390	4.90	.977	5,120	5.15	1.00	4,240						
I-6	4.35	1.39	865				4.9	.812	1,220				4.71	1.99	42
42	4.75	1.30	1,290	4.85	.81	1,810	5.1	.78	1,631	5.1	1.47	1,920	5.25	1.70	812
R5	4.45	1.45	597	5.05	.85	1,180	5.1	.68	1,160	5.05	1.37	57	4.48	1.76	36
H	4.62	1.00	447	4.9	.546	792	4.93	.596	833	5.6	.462	406	4.87	1.71	224
R2	4.32	1.35	510				4.90	.87	535				4.42	2.62	163
	*4.26	1.47	1,552	4.88	.928	2,987	5.01	.870	2,902	4.82	1.60	1,600	4.51	2.12	1,220
	**4.03	1.65	2,342	4.91	1.12	4,710	5.03	.970	5,150	4.15	2.32	2,800	4.09	2.41	2,840
	***4.49	1.29	742	4.91	.735	1,260	4.98	.747	1,070	5.28	1.13	794	4.74	3.26	255

* Average of all cultures

** Average of cultures K, 49, K4y, and 73.

*** Average of cultures I-6, 42, R5, H, R2.

TABLE VII

Percentages Increase in Counts of Lactobacillus acidophilus over that of the Control by the Addition of Calcium Carbonate and Phosphate Buffer

(Counts in Millions per c.c.)

Culture	Calcium Carbonate		Phosphate Buffer	
	5 per cent	10 per cent	1 per cent	2 per cent
K	98.4	119.7	35.3	18.9
49	40.4	11.2	34.6	91.0
K4y		254.0		15.2
73	114	77.4		
I-6		41.0		-94.2
42	41.9	26.2	49.3	-38.0
R5	18.3	94.8	-92.1	-93.9
H	77.3	86.3	-9.17	-50.2
R2		4.9		-68.0

Discussion

The results of this investigation on the use of calcium carbonate and phosphate buffer are found in Table V. It will be seen in Table V that the results have been tabulated at 12-hour intervals of the pH value, titratable acidity expressed as per cent lactic acid, and the counts of duplicate plates expressed in millions, but the six ciphers are omitted. The growth curves of the separate cultures are plotted on Graphs A, B, C, D, E, F, G, H, and I; this seems to be the most logical method of expressing the results, since this investigation was concerned with increasing the number of organisms in a culture.

From the graphs may be observed the increase or decrease in the number of organisms for the separate cultures. The results as expressed by the graphs show that in all determinations there was an increase in the number of organisms when calcium carbonate was used as the buffer substance. In many cases the results with 5 per cent calcium carbonate were higher than with 10 per cent calcium carbonate. The results with the use of the phosphate buffer were variable, as shown by the graphs. Cultures K, 49, K4y, and 42 showed an increase in count with the phosphate buffer. However, with cultures I-6, R5, R2 and H there was a decided decrease. It should be noted that cultures K, 49 and K4y are smooth strains and cultures R5, R2 and H are typical rough strains of Lactobacillus acidophilus. Culture 42 was also classified as a rough strain and showed an increase in count with 1 per cent phosphate buffer.

An average of the highest counts for the duplicate determinations of each percentage buffering material and its control are found summarized in Table VI. Also in this table is found an average of the pH values and

per cent acid at the time of highest count. In Table VII are recorded the per cent increase of the average in Table VI. The percentages found in Table VII are expressive of the results previously stated. Thus the results of this investigation show that by employing $CaCO_3$ as a buffering material the number of organisms is increased. The percentage increase, however, varies with different strains of Lactobacillus acidophilus. It should be noted at this time that the percentages in Table VII were actually higher than recorded, due to failure to correct for dilution of the original medium by $CaCO_3$. It is difficult to correct for this dilution of the medium, because of the rapid settling out of the $CaCO_3$ at the time of withdrawal of sample. However, it was found by actual measurement that the addition of 5 and 10 per cent $CaCO_3$ actually increased the volume 4 and 8 per cent respectively. The count therefore would be correspondingly higher.

The decrease in the count of rough strains R5, R2 and H and intermediate strain I-6 by the phosphate buffer is probably due to the inhibitory action of the salts. It is believed that rough strains of Lactobacillus acidophilus are more sensitive toward the media than smooth strains. This marked difference between smooth and rough strains is further indicated by observing Table V. It is noted that the maximum growth in the controls for rough strains H, R2 and R5 was in the range of pH 4.55-4.75, while that for the smooth strains was at a lower reaction of 3.8-4.2.

Another interesting fact from Table V is the low acidity at which the maximum growth is obtained. The table indicates that the highest number of organisms reached with regard to acidity was as follows: smooth strain K, 2.17 per cent; strain 49, 1.45 per cent; strain K4y, 1.26 per cent; strain 73, 1.5 per cent; intermediate strain I-6, 1.39 per cent; and rough strains 42, 1.30 per cent; strain R5, 1.45 per cent; strain H, 1.00 per cent; and strain R2, 1.35 per cent. An average of the percentage acidity at the highest counts for the smooth and rough strains was 1.65 per cent and 1.29 per cent, respectively.

GENERAL DISCUSSION

Reactions varying from pH 5.8 to 7.6 have been reported for media used for cultivation by different investigators. From the results of this investigation it would appear that the optimum reaction is limited to a narrower range since both rough and smooth strains from different sources gave higher counts on tomato juice agar at a pH 6.0-6.2. A similar effect of pH on the growth of L. acidophilus on whey agar has been noted by Black (unpublished results). These results would indicate the advisability of considering the optimum pH of the medium where maximum counts were desirable, such as in control work or commercial preparations.

Failure to obtain proper colony development may also be attributed to improper adjustment of the media. Kulp (1932) has observed that an X-type strain of L. acidophilus which developed poorly on tomato juice agar at pH 6.8-7.0 showed good colony development at 6.0-6.2. In these studies it was noted that larger colonies were obtained at the optimum reaction, particularly with rough strains.

Of several special agars suggested for the plating of L. acidophilus the most widely used are prepared from whey or vegetable extracts. Whey and tomato juice agars are obtainable in dehydrated forms. The results obtained with all cultures used in this investigation with dehydrated whey agar and prepared tomato juice agar adjusted to same pH indicate that approximately the same counts were obtained with the two media. Kulp (1927) found that approximately the same counts were obtained with tomato juice agar as with whey-galactose agar. However, dehydrated whey agar in the prepared form is adjusted to pH 6.6, thus it would seem from the

previous investigation that this was not the optimum reaction for all cultures.

Colonies on dehydrated whey, wheytone and kraftose agars were small and compact compared to the large and filamentous types of colonies on tomato juice agar. This increase in colony formation on tomato juice agar made counting less difficult. Similar observations of small compact colonies on whey-galactose and the large and filamentous type of colonies on tomato juice agar were reported by Kulp (1927) and Bachman and Frost (1932).

Increase in CO_2 up to 25 per cent have been used for the cultivation of L. acidophilus. Under the conditions employed in this investigation, an increase in CO_2 above that of the ordinary atmosphere is not necessary for the growth of all strains of L. acidophilus, especially with the smooth strains. Kulp (1926) using whey-galactose agar and employing different methods in obtaining a CO_2 tension found strains of human origin somewhat more dependent on CO_2 than strains of rat origin. Reichart and Davis (1928) using whey-peptone-galactose agar and employing somewhat the same methods used in this study found increases in the number of L. acidophilus colonies with increases in CO_2 up to 8 per cent, with variations among strains. The results of this investigation show a decrease with 12 per cent CO_2 over that of 8 per cent. Valley and Rettger (1926) found that L. acidophilus showed increased growth with increases in CO_2 in a closed chamber up to 20-25 per cent, however, no mention was made of cultures or methods used. According to the results of this study increases of 20 per cent CO_2 did not show an increase in counts over that of 10 per cent, thus, it would not appear a CO_2 tension over 20 per cent is not necessary. This inconsistency of results among various investigators may be due to failure to adjust the media to the optimum reaction for the cultures studied. Kulp

and White (1932) and Valley and Rettger (1925) observed that CO_2 increased the acidity of the media. This would tend to make growth conditions different on the control plates and plates incubated under CO_2.

Many variations have been found in the colony morphology of L. acidophilus. Investigations have shown that the typical S form of colony is the most stable type, but many variants have been observed from Rough forms of L. acidophilus colonies. The results of this study support the evidence of other investigators that the rough forms may be caused to dissociate by changes in environmental conditions. It appears that variants may be obtained by marginal fishing from the smooth growth of typical rough strains which have been continually cultivated in an atmosphere of 20 per cent CO_2. These variations, however, were not stable and tended to revert back to the original form. It may be that many of the intermediate forms found in commercial products are dissociate forms from rough strains.

In studying the effect of calcium carbonate and the phosphate buffer on the growth of L. acidophilus a close correlation was noted between acidity and the number of organisms. When an acidity of less than 1 per cent was maintained by the addition of 5 or 10 per cent calcium carbonate, the maximum growth was obtained. This is supported by the findings of Black (1931) who found that the maximum growth in milk cultures was obtained at an acidity of one per cent or less, with some variation among strains. The work of Kopeloff, Etchells, and Kopeloff (1934) also show that the viability of L. acidophilus is closely associated with acidity, and that higher counts are obtained with smooth than with rough strains. In this work it was found in the control cultures that smooth strains of L. acidophilus produced approximately 4 times higher counts than rough strains with some

variation among strains. One smooth strain gave a count of 3470 million in the control and counts of 7270 million and 7885 million when 5 per cent and 10 per cent calcium carbonate, respectively, were added. However, it was observed that the per cent increase in acidity was not in proportion to the increase in counts. Smooth strains produced on an average of 1.65 acid and a count of 2,342 million, while rough strains produced an average of 1.3 per cent acid and a count of 742 million. When calcium carbonate was added the acidity was not increased over 1 per cent until the count reached approximately 2000 million organisms, a condition which occurred only among the smooth strains, as cultures of rough strains failed to obtain such high counts. Maximum growth in the control cultures and cultures containing calcium carbonate was at the end of 72 to 84 hours incubation. The phosphate buffer was not as effective as calcium carbonate in increasing the count because of the increase in acidity produced by the buffer after 12 hours incubation. Rough cultures which were found to be more sensitive to the increase in acidity than smooth strains were shown by the decided decrease in the number of organisms of cultures from rough strains after 24 hours incubation. From the results it would appear that a phosphate buffer would not be effective in increasing the growth of cultures from rough strains. Increasing the buffer salts to three per cent resulted in coagulation of milk and a complete inhibition of growth after 12 hours incubation.

SUMMARY

The optimum reaction for the growth of all cultures of Lactobacillus studied was pH 6.0-6.2. Rough strains showed a decided decrease in count when the medium was adjusted to pH 5.8 or 6.4. Counts on the medium were adjusted to pH 5.8 or 6.4. Counts on plates from smooth strains were found to be, in many instances, as high at pH 5.8 and 6.4 as at 6.0 and 6.2. In no instances were counts higher than at pH 6.6.

Counts of L. acidophilus obtained with Bacto dehydrated whey agar and tomato juice agar of the same pH were approximately the same for all cultures studied. Tomato juice agar gave larger and more characteristic colonies. Tomato juice agar is easily prepared and the resulting medium is a clear, light-brown product. The main difficulty encountered with tomato juice agar was controlling of pH during sterilization. Bacto dehydrated whey agar in the prepared form has a reaction of pH 6.6; from the results obtained on pH of medium this would not seem to be the optimum reaction for all cultures.

Agar prepared from the respective sweet whey powders, wheytone and kraftose, gave counts from 5 to 7 strains comparable to those obtained with tomato juice agar. Counts with 2 strains were approximately 20 per cent higher on wheytone and kraftose agars than on tomato juice agar. Wheytone and kraftose agars when properly filtered during preparation resulted in a clear media.

Of the fourteen strains of L. acidophilus studied, six showed an increased growth on tomato juice agar adjusted to the optimum reaction when incubated in an atmosphere of 10 per cent CO_2. Some strains were apparently influenced more than others. This increase in growth was

among the rough strain. Three of the nine rough strains, the intermediate strain and the smooth strains, were not influenced by the increased presence of CO_2. An atmosphere of 20 per cent CO_2 showed the same influence in the count as 10 per cent CO_2, thus it would appear that a CO_2 tension over 10 per cent would not be necessary.

Colony formation was influenced by different growth factors. The optimum reaction for the growth of all cultures on tomato juice agar was characterized by an increase in the size of the colony. Tomato juice agar gave larger and more characteristic colony formation than Bacto dehydrated whey, wheytone, and kraftose agars when the media were adjusted to the optimum pH and incubated without an increased presence of CO_2. However, colonies on wheytone and kraftose agars were larger than colonies on Bacto dehydrated agars. Increased growth in presence of CO_2 was also associated with an increase in the development of the surface colonies on tomato juice agar adjusted to the proper reaction.

The intermediate strain and rough strains showed a tendency to show smooth colony development on the surface of tomato juice agar of optimum pH when incubated in the presence of CO_2. By selection of smooth type colonies and marginal fishings variants were observed after the third repeated isolation and replating on tomato juice agar and incubated under 20 per cent CO_2. These variants of the rough strains were small, flat surface colonies and compact subsurface colonies on plates which were incubated under CO_2. Surface colonies were not observed on plates incubated in ordinary atmosphere, while the subsurface colonies were small and compact. These variants were not stable and tended to revert to the original R forms when incubated under ordinary atmospheric conditions.

The addition of 5 per cent and 10 per cent calcium carbonate to milk cultures caused an increase in the number of L. acidophilus obtained. Five

per cent calcium carbonate was found to be as effective in most determinations toward increasing the numbers as 10 per cent calcium carbonate. Increases with the smooth strains varied from 40 to 254 per cent. With rough strains the increase varied from 4.9 to 98.4 per cent. Five and ten per cent calcium carbonate maintained an acidity of less than 1.2 per cent in practically all determinations with an average acidity of less than .85 per cent.

The results obtained by the addition of one per cent and two per cent phosphate buffer to milk cultures of L. acidophilus were variable. The two cultures from smooth strains which contained 1 per cent buffer showed an increase in numbers of 35 per cent. The three cultures which contained 2 per cent buffer showed increases of 15.2, 18.9, and 91 per cent. The intermediate strain and 4 rough strains showed a decrease with one and two per cent buffer. In most of the determinations the per cent acidity was increased on addition of buffer.

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