A COMPARISON OF VARIOUS INCUBATION TEMPERATURES FOR THE PRIMARY ISOLATION OF COLIFORM ORGANISMS FROM WATER

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INTRODUCTION

In the routine bacteriological examination of water for the presence of organisms of the coliform group, the accepted presumptive fermentation test involves the inoculation of lactose broth with suitable aliquots of the water sample, followed by incubation at 37° C. The optimum temperature for the growth of B. coli (1) and other organisms of intestinal origin has been considered to be 37° C., and hence primary isolation is attempted at this "optimum temperature". The question arises whether the optimum temperature for the growth of coliform organisms in their normal habitat is also the optimum growth and fermentation temperature after those organisms have existed in water for some time at temperatures normally much lower than 37° C.

A review of the literature reveals no instances where presumptive fermentation tests were attempted at temperatures lower than 37° C., however a few investigators have tried primary isolation at temperatures as high as 46° C. For example, Minkevich (2) in applying the principle of Eijkman's (3, 4, 5) fermentation test for determining the "coli titre" of water, found that at temperatures of 46°, 45°, 44°, and 43° maximum dilutions of sewage polluted water and suspensions of coli cultures are not always able to ferment mannitol, being inhibited more by the higher temperatures. Nevertheless, Minkevich and his workers concluded that 43° C. is the highest temperature harmless for coli in "maximum dilutions" of water samples, although higher fermentation titres were obtained when 37° C. was used as the incubation temperature. Other investigators, unlike Minkevich, employed pure cultures of intestinal organisms isolated from various sources in testing the efficacy of tem-

peratures higher than 37° C. for the primary isolation and detection of organisms of fecal origin. Sherwood and Clegg (6, 7) contended that the most reliable test for the detection of "Bacterium coli" was incubation in MacConkey's broth at 44° C. Experimental results obtained with pure cultures of organisms isolated from shellfish, sewage and feces were offered in support of their argument. However, conclusions drawn from experimental results obtained with pure cultures grown at the normally optimum temperatures are not necessarily valid for the primary isolation or detection of those same organisms after they have existed for some time in a sub-optimum temperature environment.

Intestinal organisms that survive for extended periods in water at temperatures that are usually much lower than 37° C. conceivably might become so adapted to those lower temperatures that a relatively sudden return of the organisms to the temperature of their normal environment could, in many cases, seriously impair their ability to survive and multiply. Therefore, this investigation was undertaken to determine the optimum temperature for the primary isolation of coliform organisms from water.

Four specially constructed incubators, each with a capacity of one cubic foot, were used throughout these studies. These incubators were carefully controlled to maintain the desired T° C. ± 0.5°, and were regulated to 32°, 35°, 37° and 39° C., respectively.

The first phase of the experimental work involved growth curve determinations at the above four temperatures for important representatives of the coliform group. All of the organisms (13 in number) thus studied had been isolated in pure culture from various water sources. The purpose of these growth curve runs was to ascertain whether there were sig-

nificant differences in the growth rates of the various coliform types at the temperatures under consideration. It should be emphasized that since their initial isolation from water, all sub-cultures of these test organisms had been incubated at 37° C.

The second and perhaps most important phase of the experimental procedure involved actual presumptive fermentation tests of a series of water samples obtained from a small lake near Lansing, Michigan during the months of August and September, 1948. In order to obtain as great a variety of coliform organisms as possible, the samples were collected in the customary manner from all parts of the lake. No more than twenty samples were taken at any one time, since that figure represented the limited capacity of the test incubators. Within 24 hours after collection, the presumptive tests were performed concurrently at 32°, 35°, 37°, and 39° C. upon each sample of water. In every case where the presumptive fermentation test was positive, an attempt was made to isolate the organism or organisms responsible for the gas production.

EXPERIMENTAL PROCEDURE

A. Growth Curves

The following organisms were used in the growth rate studies:

Escherichia coli -4 cultures, Nos. 2, 5, 7 and 8.

Aerobacter aerogenes - 4 cultures Nos. 25, 26, 27 and 29.

Intermediate I (- + - +) -5 cultures, Nos. 29, 31, 32, 33 and 35.

Cultures of the above organisms were carried on plain agar slants. Uniform saline suspensions of the organisms were prepared from 18 to 24 hour single strength lactose broth cultures that had been incubated at 37° C. The saline suspensions were so diluted that when 1 ml. of a

suspension was added to 99 mls. of single strength lactose broth in an Erlenmeyer flask, the number of organisms present per ml. in the flask did not exceed 100. The flasks were machine-shaken for five minutes to insure unfform distribution of the organisms. 15 ml. aliquots from each broth culture thus prepared were pipetted into each of four sterile test tubes. After plating out 1 ml. from each tube to determine the initial counts, the tubes were distributed among the four incubators set at 32°, 35°, 37° and 39°, respectively. At the end of 2, 4, 6, 8, 12 and 24 hours 1 ml. aliquots from each tube were plated with tryptone-glucose-extract agar. All plates were counted after 48 hours incubation at 37°.

At the same time the 15 ml. portions of the broth cultures were pipetted into sterile tubes, 1 ml. aliquots were added to each of four Durham single strength lactose fermentation tubes which were then incubated with the other cultures, one at each temperature. Gas production in percent was read and recorded for each incubation temperature after 12, 24 and 48 hours.

A total of 29 such determinations were made. Three of the E. coli cultures were run twice, and one was run three times. The four A. aerogenes cultures were handled similarily. Of the five Intermediate I. cultures, four were run twice and one three times.

Following the accepted procedure for handling such data, the logarithmic average counts were determined for each coliform type, the data for which are given in Tables I, II, and III. The logarithmic growth curves were plotted from those data, and are represented by Graphs I, II and III.

TABLE I

A Comparison of Various Incubation Temperatures for *E. coli**

Log Average Counts

Time in Hours	Incubation Temperature °C.			
	32°	35°	37°	39°
0	1.78	1.79	1.81	1.80
2	2.44	2.63	2.77	2.65
4	3.73	4.30	4.63	4.74
6	5.36	5.85	6.33	6.34
8	6.65	7.41	7.72	7.82
12	8.31	8.63	8.57	8.61
24	8.80	8.72	8.57	8.51

Average Gas Production

Time in Hours	Incubation Temperature °C.			
	32°	35°	37°	39°
12	4%	14%	8%	14%
24	24	33	31	32
48	32	36	34	34

*4 cultures (Nos. 2, 5, 7 and 8) - 9 runs

TABLE II

A Comparison of Various Incubation Temperatures for A. aerogenes*

Log Average Counts

Time in Hours	Incubation Temperature ° C.			
	32°	35°	37°	39°
0	1.77	1.74	1.75	1.76
2	2.33	2.53	2.63	2.94
4	3.98	4.35	4.48	4.54
6	5.36	6.35	6.31	6.32
8	6.93	7.67	7.64	7.62
12	8.47	8.51	8.13	8.31
24	8.89	8.71	7.42	7.20

Average Gas Production

Time in Hours	Incubation Temperature ° C.			
	32°	35°	37°	39°
12	6%	8%	10%	7%
24	38	40	32	26
48	51	51	36	29

* 4 cultures (Nos. 25, 26, 27 and 29) - 9 runs.

TABLE III

A Comparison of Various Incubation Temperatures for Intermediate I*

Log. Average Counts

Time in Hours	Incubation Temperature °C.			
	32°	35°	37°	39°
0	1.70	1.71	1.70	1.69
2	2.12	2.21	2.23	2.15
4	3.51	3.52	3.15	3.02
6	4.71	4.92	4.41	4.07
8	6.07	6.43	5.54	5.33
12	8.02	8.17	6.61	6.55
24	8.71	8.61	7.17	6.24

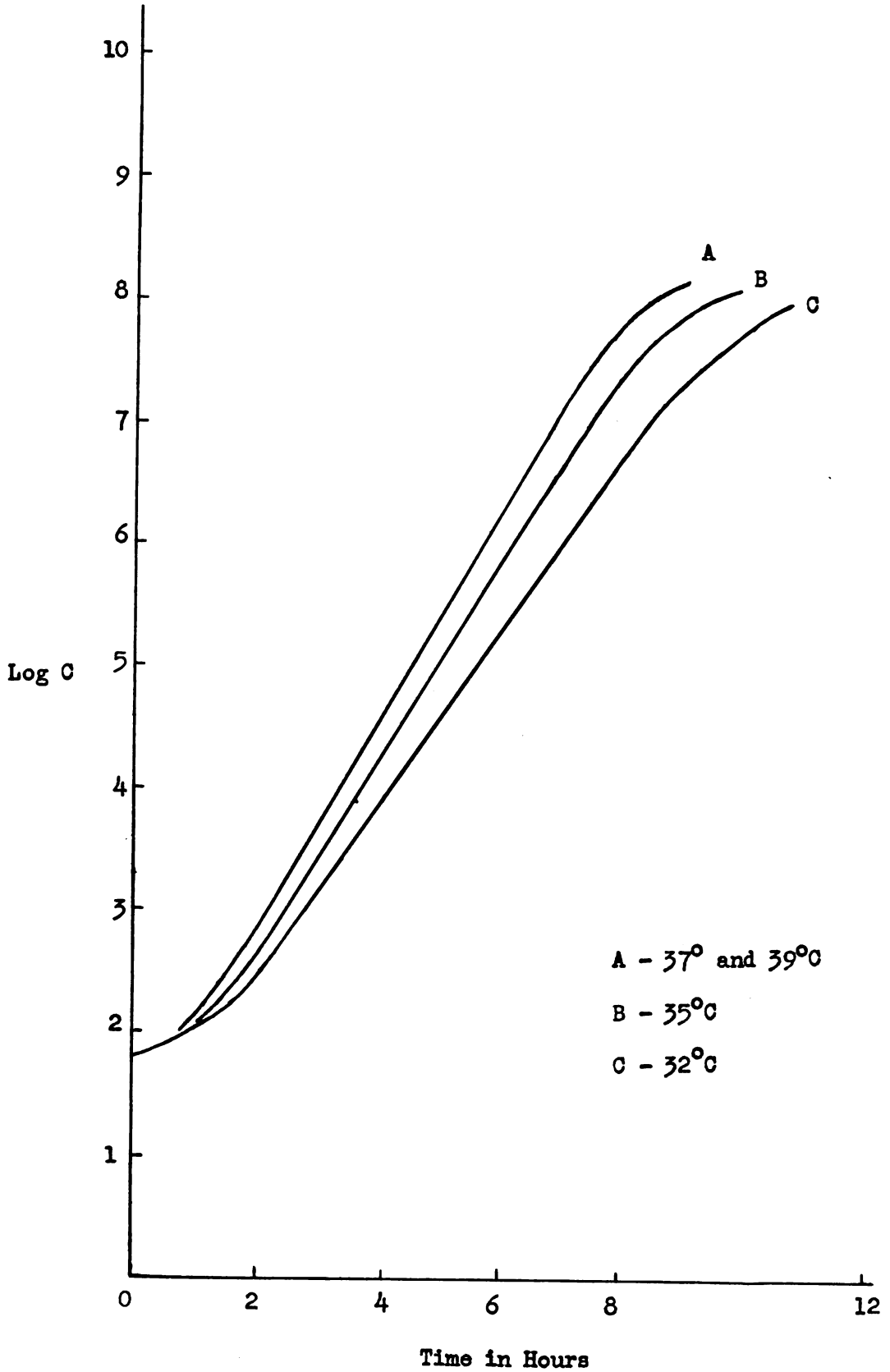
Average Gas Production

Time in Hours	Incubation Temperature ° C.			
	32°	35°	37°	39°
12	0%	0%	0%	0%
24	5	11	5	3
48	23	27	11	7

*5 cultures (Nos. 29, 31, 32, 33 and 35) - 11 runs.

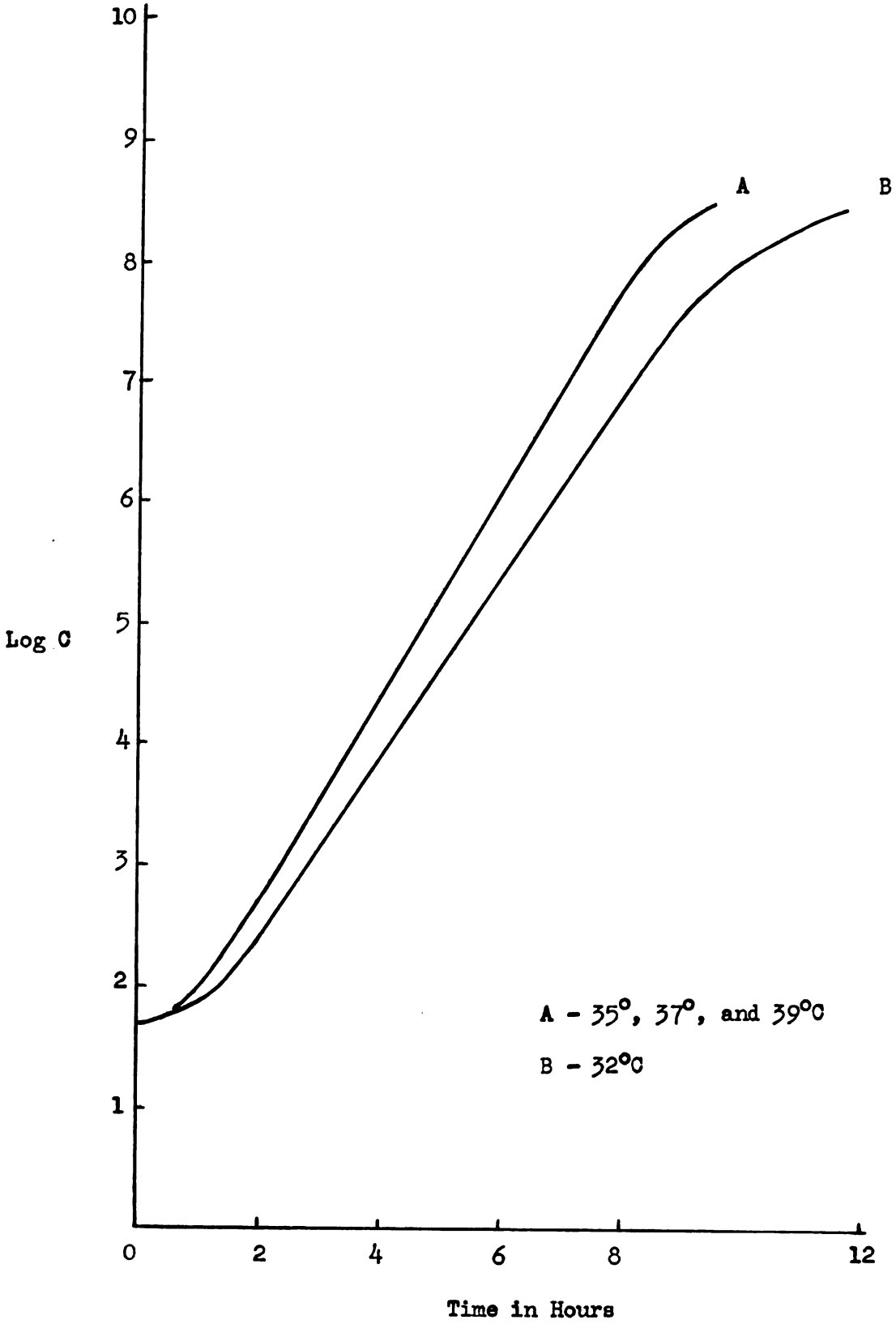
GRAPH I.

Growth Curves for <u>E. coli</u> at Various Incubation Temperatures



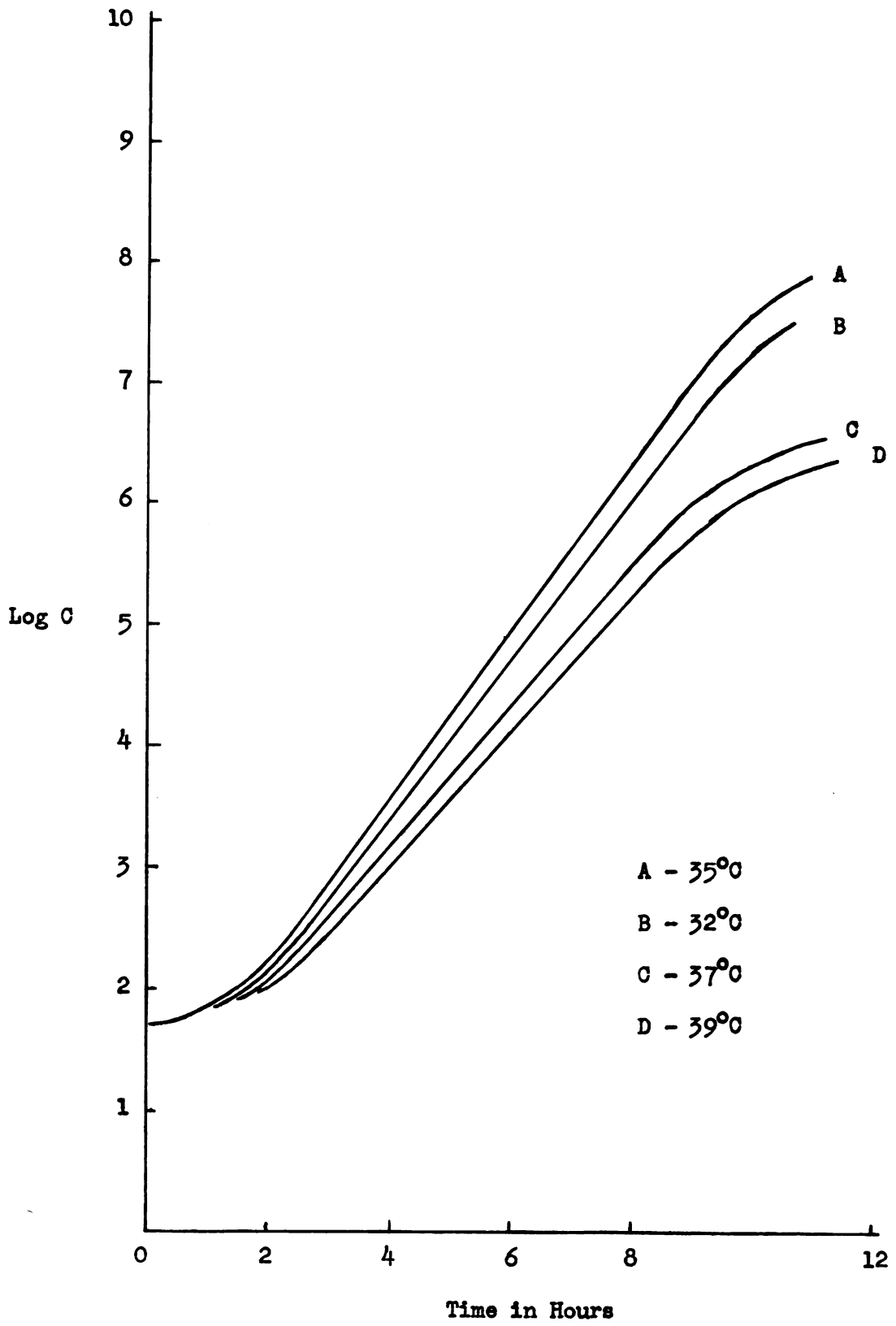
GRAPH II.

Growth Curves for A. aerogenes at Various Incubation Temperatures



GRAPH III.

Growth Curves for Intermediate I. at Various Incubation Temperatures



B. Water Samples

Of the 158 water samples which produced gas in lactose broth within 48 hours, 147 of them were successfully confirmed by isolation of the coliforms responsible for the fermentation.

A modified, 4 - tube presumptive fermentation test was performed on each water sample at each of the four incubation temperatures. In every case, 10 mls. were added to each of two double strength lactose fermentation tubes, and 1.0 ml. and 0.1 ml. were added, respectively, to two single strength lactose fermentation tubes. All tubes were examined for gas production, which was recorded in percent for each positive tube, at the end of 12, 24, and 48 hours.

Isolation of the coliforms was attempted by streaking Levine eosine methylene blue agar plates from those tubes showing the maximum gas production after 48 hours for each sample. After 24 to 48 hours incubation at 37° C., morphologically distinct, suspect colonies were picked from each E.M.B. plate and transferred to plain agar slants. The lactose fermenting ability of each culture thus isolated was verified before carrying out the Imvic reactions. As recommended by Standard Methods, Kovacs' (8) modification of the indol test, Barrit's (9) modification of the Voges-Proskauer reaction, and Koser's (10) procedure for the citrate test were employed. Whenever the Imvic reactions tallied + + + + or + + + + the purity of the cultures responsible for them was checked. Altogether only three types of intermediates were isolated, i.e., those giving - + - +, + + + +, and + + + + Imvic reactions, respectively.

BLE IV

Data for Representative [illegible] Showing Number of Positive Presumptive Tubes, Maximum Gas Production [illegible] Isolated at the Various Incubation Temperatures

Water Sample No.		Incubation Temperature 35° C. Hours			37° C. Hours			39° C. Hours		
					12	24	48	12	24	48
						50	3-60	0	2-50	2-60
							2-30	0	1-20	2-40
				2-50	0	0	1-30	0	0	2-B
				70	0	1-50	1-50	0	1-20	1-70
				-35	0	0	0	0	1-70	2-80
				5	4-60	0	0	1-B	0	1-50	2-45
					2-70	0	1-55	2-70	0	1-10	1-60
				-B	2-45	3-60	0	0	1-20	0	0	1-45
111	coli			0	2-40	2-40	0	0	1-35	0	0	0
125	- + + +	1-B	2-8	2-90	2-B	2-10	2-60	1-B	1-5	1-5	0	1-30	2-45

NOTE: The first number indicates the number of tubes (4 is the maximum possible) showing gas, and the second number represents the maximum gas production in percent.

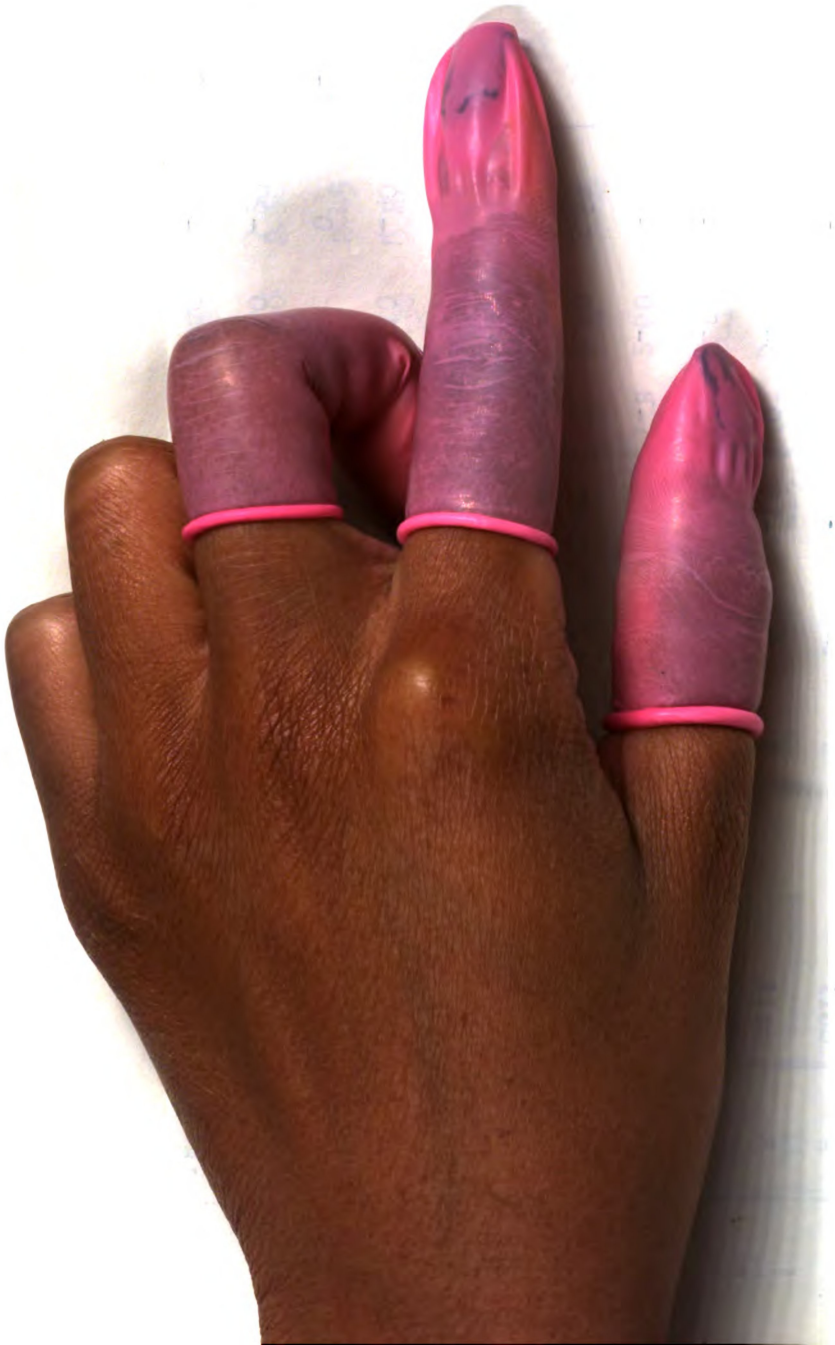


Table V

The Effect of Incubation Temperatures on the Total Number of Tubes* Showing Gas for All Water Samples

Coliform(s) Isolated	No. of Samples	Incubation Temperature 32° C. Hours 12	24	48	35° C. Hours 12	24	48	37° C. Hours 12	24	48	39° C. Hours 12	24	48
coli	39	7	49	89	15	65	86	7	42	64	16	47	68
aerogenes	62	22	75	148	30	107	151	21	73	101	30	92	123
coli & aerogenes	23	6	35	57	10	38	49	11	31	39	15	38	45
intermediate	15	2	17	28	5	19	32	3	19	23	6	20	25
coli & intermed.	2	1	2	3	1	2	4	0	0	2	1	2	3
aerog. & intermed.	3	0	2	7	1	5	6	0	0	2	0	4	5
coli, aerog. & intermed.	3	1	3	6	2	2	7	3	4	4	2	4	7
TOTAL	147	39	183	338	64	238	335	45	169	235	70	207	276

*Maximum of 4 tubes per sample at each temperature.

TABLE VI

The Effect of Incubation Temperatures on the Number of Samples Giving Positive Presumptive Tests.

Coliform(s) Isolated	No. of Samples	Incubation Temperature											
		32° C.			35° C.			37° C.			39° C.		
		Hours			Hours			Hours			Hours		
		12	24	48	12	24	48	12	24	48	12	24	48
coli	39	6	30	38	12	36	38	6	26	34	11	29	36
aerogenes	62	15	47	62	22	59	62	16	44	53	22	51	59
coli & aerogenes	23	5	21	23	9	20	22	10	17	18	9	19	22
intermediate	15	2	12	14	3	14	15	3	11	13	5	13	14
coli & intermed.	2	1	1	2	1	1	2	0	0	1	1	2	2
aerog. & intermed.	3	0	2	3	1	3	3	0	0	1	0	3	3
coli, aerog., & intermed.	3	1	3	3	1	1	3	3	3	3	1	3	3
TOTAL	147	30	116	145	49	134	145	38	101	123	49	120	139

TABLE VII

Average Maximum* Gas Production for All Positive Samples at the Various Incubation Temperatures

Time	32° C.	35° C.	37° C.	39° C.
24 hours	5.3%	23.5%	29.4%	28.6%
48 hours	40.3%	56.7%	39.1%	47.9%

*In calculating the average maximum gas percentages, a bubble of gas was arbitrarily considered as 2%.

A complete tabulation of the data for a number of the water samples thus tested is given in Table IV. A compilation of all the pertinent data obtained is given in Tables V, VI and VII.

DISCUSSION

From a study of the logarithmic growth curves obtained for both E. coli and A. aerogenes, it is evident that their optimum growth temperature must lie in the 37° to 39° C. range, since the growth curves plotted for either organism at those two temperatures are practically coincident. However, there appears to be no marked decrease in the growth rates of E. coli and A. aerogenes when they are incubated at 35° C.

Also, it is apparent that the optimum growth temperature for Intermediate I. is quite definitely close to 35° C., even though that organism is only slightly inhibited by a temperature of 32° C. On the other hand incubation at 37° or 39° C. appreciably decreases its rate of growth, as is obvious from the much lower bacterial populations at the stationary phases for those two incubation temperatures.

The data tabulated in Tables V and VI are most illuminating. Since the total number of tubes showing gas for all samples proved to contain coliforms is appreciably greater at both 32° and 35° C., than at 37° or 39° C., it is evident that the optimum or critical temperature for the primary isolation of coliform organisms from water is less than the temperature of their normal environment. Further, it appears that the best incubation temperature for the primary isolation of coliforms from water is closest to 35° C., since the greatest number of tubes showed gas after 24 hours incubation at that temperature. However, longer incubation at 32° C. produced equally good results. These observations are further substantiated by a consideration of the number of confirmed pos-

itives obtained for the various incubation temperatures, which data are given in Table VI. Although 32° and 35° C. produced the same number of confirmed positive presumptive tests after 48 hours, again 35° C. seems to be the best, since the most positives after 24 hours incubation are recorded for that temperature.

A further study of the data with regard to a comparison of the type or types of coliforms isolated with the results obtained at the four incubation temperatures is also very interesting. The data clearly testify to the undesirability of subjecting both E. coli and A. aerogenes to a relatively sudden return to the temperature of their normal environment, after those organisms have sojourned for some time in water at temperatures far below the normal optimum. There is a striking correlation in each case between the total number of tubes showing gas, the total number of positive presumptives, and the incubation temperatures which heavily favors the two lower temperatures, especially 35° C. One can only conclude that the normal optimum temperatures often has a definitely harmful influence upon the ability of certain coliforms to survive and multiply, when it is applied for their primary isolation from water. The data obtained for those water samples from which only intermediates were isolated are also suggestive of this viewpoint, although not so conclusively, since relatively few such samples were encountered in the series tested.

An examination of the percentage gas production data (Tables I, II and III) accumulated during the pure culture studies reveals some tendency (least remarkable for E. coli) for greater gas production at the lower temperatures, especially 35° C., in the case of each coliform

type. This is true even though that temperature can be correlated with the growth curve optimum temperature only in the case of Intermediate I. Although a study of the average maximum percent gas production data at the four temperatures, given in Table VII, for all 147 positive water samples is equally inconclusive, there again is some suggestion that 35° C. may be the optimum fermentation temperature.

The results of the pure culture studies in no way hint as to the probable outcome of the more practical water sample tests which followed. Thus the importance of using "water-adapted" organisms, and hence actual water samples, rather than pure cultures for a study of this nature is evident.

CONCLUSIONS

1. The normal optimum growth temperature for Escherichia coli and Aerobacter aerogenes is in the 37° to 39° C. range.

2. The normal optimum growth temperature for Intermediate I. is approximately 35° C.

3. The most favorable incubation temperature for the primary isolation of coliforms from water is closest to 35° C.

4. Incubation at 32° C. for primary isolation is also superior to 37° C., although a longer incubation period is necessary at that temperature than at 35° C.

5. Data are presented which suggest that 35° C. may be the optimum fermentation temperature for coliform organisms.

It is recommended that 35° C. be accepted as the incubation temperature for presumptive tests for coliforms in water, inasmuch as that temperature is now officially recommended (11) for determining plate counts in milk.

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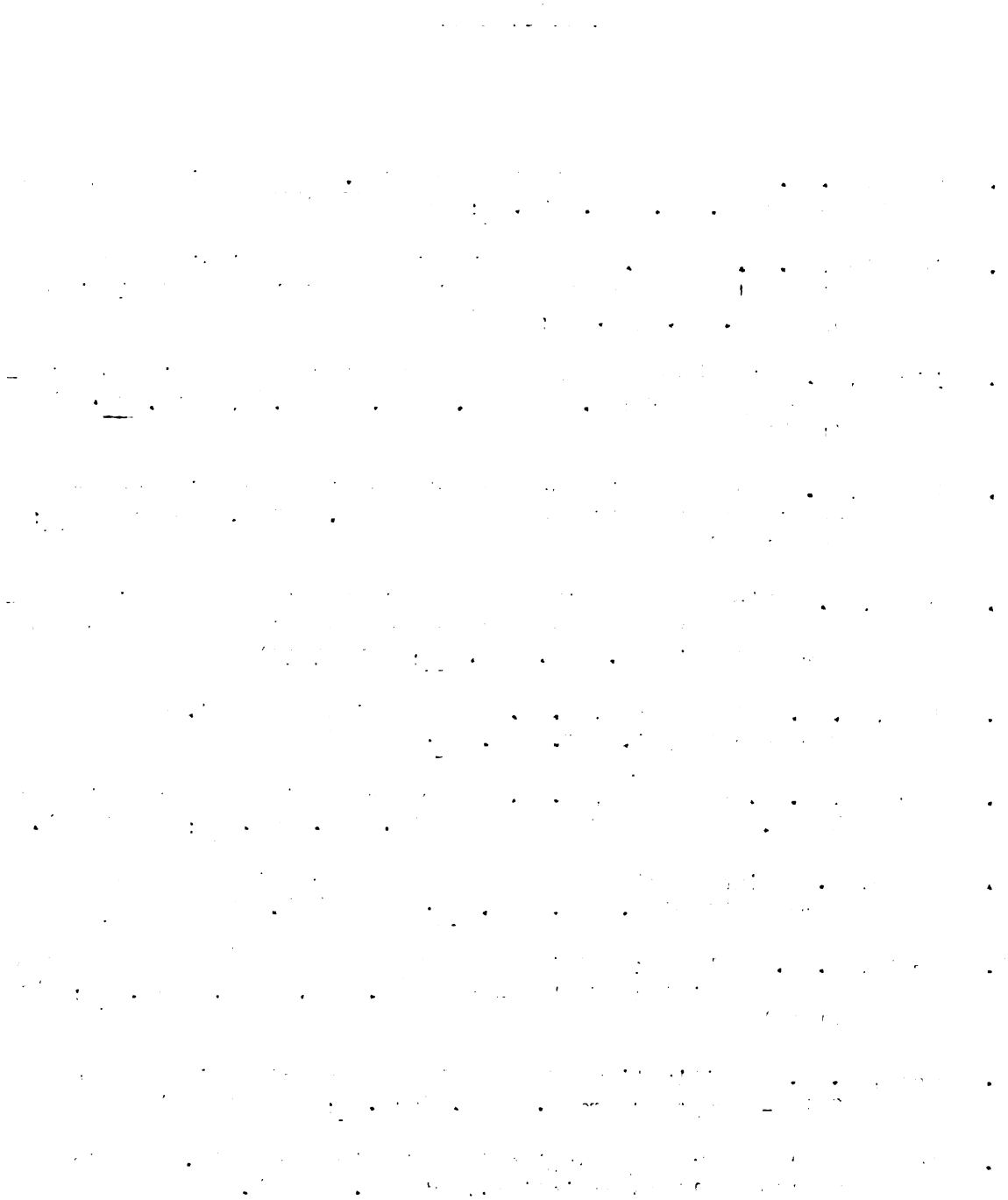

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