INFLUENCES AFFECTING THE GROWTH OF MICRO-ORGANISMS — LATENCY: INHIBITION: MASS ACTION.¹

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I. INTRODUCTION.

THE method of detection and estimation of the various kinds of *Bacillus coli* in water samples sent for examination to this laboratory does not differ materially from that followed elsewhere, save in the particular that, as a preliminary, suspicious organisms are not submitted to further tests unless there is first produced on Grünbaum's modification of MacConkey's medium (neutral-red taurocholate lactose agar) one or more colonies of a yellowish, yellow-red, or reddish tinge.

Such colonies are isolated and emulsified in peptone water or broth, which is then added to various sugar broths, including among others dulcitate, lactose, and glucose.

With organisms which prove subsequently to be of the *B. coli* type acid fermentation, with foaming, of these various broths sets in at different intervals: glucose broth usually first of all, later lactose broth; and, as a rule, last of all, at varying intervals, dulcitate broth. These specific fermentation tests are conducted at blood heat; with dulcitate broth a duplicate test is made at room temperature (20° C.). The period after which dulcitate broth ferments is usually two to three days, at 37° C.; at 20° C. three to four days elapses. Rarely at 37° C. dulcitate broth fermentation may take place overnight; sometimes the interval is more than seven days. Occasionally it has happened that dulcitate broth has fermented at the lower temperature, at varying intervals preceding the fermentation at blood heat, and rarely the fermentation test, while positive at the lower temperature, has failed altogether at the higher temperature. Exigencies of time do not usually permit these tests to be followed for more than eleven days. If subcultures be made from a fermenting dulcitate broth to fresh dulcitate broth, as a rule positive fermentation sets in within twenty-four hours at 37° C., within forty-eight hours at 20° C.,—this despite the period elapsing in the first test before acid production and foaming became evident. Continued subculturing does not further lessen the period elapsing before the reaction becomes positive. Sometimes in all

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these preliminary tests the dulcete broth appears to remain unchanged until a few hours preceding the reaction, when a slight opalescence or turbidity may be noticed; at other times the turbidity may appear within a few hours from the period of inoculation, and remain unaltered until acid fermentation with foaming occurs.

The delay in the appearance of a specific physical change in a nutrient medium due to the vital activity of organisms may be noted and reduced by similar methods of subculture. Thus with the laboratory variety of *Bacillus tetani* which is occasionally propagated by anærobic subculture from glucose-litmus broth, inoculation on to gelatin is followed after a period of ninety days by the appearance of liquefaction of the medium; on further subculture from the liquefied medium to fresh gelatin, liquefaction occurs on the sixteenth day. Further subculture on to fresh gelatin did not reduce the period elapsing before the commencement of liquefaction. The initial dose of inoculating medium in this instance did not appear to affect the time of onset of liquefaction; nor in the action of the varieties of *B. coli* on dulcete broth does the increase of the inoculating dose in the first instance appear substantially to hasten or delay the onset of acid production.

An instance of the delay and subsequent hastening in the appearance of a specific pigment occurred in connection with a specimen of the bacillus of blue pus. On subculture at 20° C. on agar a growth occurred within three days having characters indistinguishable from those associated with bacillus pyocyaneus. The characteristic pigment did not appear before the tenth day, but on subculture the pigment became easily recognisable on the third day.

Of a different order would be the delay in the appearance of diphtheria bacilli in a subculture made from a throat-swab on to inspissated blood serum. Usually a plentiful growth occurs overnight on incubation at blood heat. Occasionally, however, no growth is visible till the second day; and an instance occurred of a delay of forty-eight hours in the appearance of the growth. In all these cases further subcultures on to fresh serum media yielded an abundant growth overnight on incubation at blood heat.

It seems clear, therefore, that the rate of growth of an organism, or the development of certain associated physical characteristics, may be affected materially by a change in the environment, and that the previous history of an organism may give the clue and perhaps the explanation of its vagaries when a change of pabulum or other surrounding occurs.

II. GROWTH OF *BACILLUS COLI*.

In order to elucidate some of these phenomena the following experiments were undertaken.

The variety of *B. coli* used is that obtained from Escherich's laboratory. It has been propagated once a fortnight for several years by subcultures on to fresh agar, incubated overnight at 37° C., and subsequently kept in a dark cool cupboard. From a subculture on to neutral-red, taurocholate agar was picked out a single red colony, and further subcultures made as required.

The rate of growth in various media was determined by method of

plating at frequent intervals with known dilutions. Each result shown is the average of at least five plates; while for the milk experiments, at least six plates were used for each estimation.

In the graphs showing the rate of growth the ordinate is in terms of the logarithm to the base 2 of the number present per cubic centimetre. This method of representation facilitates all calculation as to the time taken at any particular portion of the experiment for the organisms present to double their numbers, and forms the groundwork for the direct measurement of an adverse influence.

1. Growth of *B. coli* in Peptone Water.

(Peptone, 1 per cent. + NaCl, $\frac{1}{2}$ per cent. + Water : sterilized.)

Trial experiments show that both at 37° C. and at 20° C., after twelve hours' incubation of peptone water inoculated with *B. coli* from a parent peptone-water culture, the rate of growth of the organism is maximal.

After a series of subcultures of *B. coli* in peptone water extending over several weeks in order to eliminate all adverse factors due to previous history of the organism, a test peptone water culture is made and incubated at 20° C. At the fourteenth hour a fresh subculture is made, and with the precautions of evenly distributing the organisms in the fluid by agitation, estimations of the number present are made at frequent intervals.

The following is the result:—

TABLE P., corresponding with CURVE P (CHART I. p. 25).

Age of Parent Culture.	Age of Subculture.	No. of Coli per c.c.	Log ₂ .
14½ hours.	0 hours	130	7·02
	1¾ „	200	7·64
	2¾ „	370	8·53
	3¾ „	590	9·23
	4¾ „	1,080	10·07
	7¼ „	4,500	12·13
	9¾ „ *	14,000	13·77

* Turbidity appearing.

Here growth is immediate. The full development is marked at about three and three-quarter hours. The maximal growth, however, does not appear from this table or from Curve P before the third hour. The restraint at the beginning is but slight, and under no other condition of experiment is there so little restraint. To emphasise this point an experiment was conducted by inoculating peptone water from a parent peptone water culture of *B. coli*, seventy-two hours old; eight hours later the experiment is repeated.

TABLES PP1 and PP2, incorporated in CURVE PP (CHART I. p. 25).

	Age of Parent Culture.	Age of Subculture.	<i>B. coli</i> per c.c.	Log ₂ .
PP1.	72 hours . .	0 hours	290,000	18.14
		2 "	347,000	18.40
		4½ "	390,000	18.57
		6½ "	742,500	19.50
		8 "	2,300,000	21.18
		11½ " *	16,250,000	24.25
		14½ "	48,333,000	25.52
		26½ "	147,500,000	27.13
		29½ "	219,500,000	27.71
		32 "	224,000,000	27.74
PP2	80 hours . .	50½ "	315,000,000	28.83
		0 hours	182,500	17.47
		3½ "	230,000	17.81
		6½ "	653,000	19.31
		18½ " *	79,250,000	26.24
		22 "	132,000,000	26.97
		24½ "	145,250,000	27.11
		43 "	241,750,000	27.85
		72 "	335,000,000	28.32

* Turbidity.

Here the rate of growth is markedly delayed for the first four and a half hours. There is some improvement at hours four and a half to six and a half, and thereafter the rate of growth appears to be maximal.

Had the rate of growth been maximal from the beginning, the numbers present at hour six and a half would have been reached between the second and third hours following inoculation. Hence the factor of restraint may be measured in terms of growth as about four hours, and assuming the organisms to double their number hourly, the measure of restraint is roughly = 4.

Still a further experiment was conducted, by inoculating peptone water with *B. coli* from a parent peptone water culture at 398½ hours, with results:—

TABLE PPS, corresponding with CURVE PPS (CHART I. p. 25).

Age of Parent Culture.	Age of Subculture.	No. per c.c.	Log ₂ .
398½ hours . .	0 hours	390,000	18.57
	5 "	500,000	18.93
	8 "	2,225,000	21.08
	11 "	15,000,000	23.84
	14 "	46,000,000	25.45

Here there is a marked restraint of growth for the first five hours; hours five to seven and a half there is a marked acceleration; finally, growth becomes maximal. By direct measurement from the graphic representation the total restraint of growth is about four and a half to five hours maximal growth.

Thus, comparing experiments P, PP, and PPS, there is, in every case, growth directly following inoculation of the new medium.

The restraint in growth as measured from the graphs on Chart I. is as follows:—

TABLE L.

	Experiment.		
	P.	PP.	PPS.
Age of parent culture .	14½ hours	76 hours *	398½ hours
Restraint of growth in terms of maximal development. }	1 hour	About 4 hours	About 4½ hours

* Mean of two experiments.

Thus, deducting the factor of minimised restraint, it becomes evident that with a stale peptone water culture of *B. coli* certain conditions must be developed which so impair the vitality of the organisms present as to restrain, for a time, their normal power of development when placed under the most favourable conditions for growth. This impairment of vitality appears to vary somewhat directly with the age of the parent culture. With a *B. coli* culture in peptone water grown at 37° C., and thereafter kept at that temperature, this impairment of vitality of the organisms present becomes marked to such an extent that the power to multiply is entirely lost; death of such organism may therefore be inferred.

It might be considered that prolonged detention of organisms in a medium loaded with their waste products of activity—at a temperature which normally is associated with their maximal rate of growth—is tantamount to their starvation and intoxication, with death of the least resistant. If the stale culture be exposed to 0° C., a temperature at which normally no growth is known to occur, then it may be presumed that metabolism is at its minimum, and that the amount of intoxication is likewise lessened,—with the result that fewer organisms would be destroyed.

That such is the case is shown from the following table, SF:—

Date of Experiment, 1908.	No. of Organisms per Cent. Volume.		
	Age of Culture.	At 37° C.	At 0° C.
May 11	5 days	302	...
" 12	6 "	205	...
" 13	7 "	195	...
" 14	8 "	190	...
" 18	12 "	148	→ 148
" 19	13 "	82	142
" 22	17 "	25	95
" 25	20 "	10	64
June 10	41 "	2·5	11

On 18th May the culture at 37° C. was subdivided and one half kept at 0° C. An enormous difference in the rate of decrease in the refrigerated portion becomes apparent.

It might be argued that the decrease in the refrigerated portion is still

due to the toxic action of the waste products contained in the medium; but this contention is negatived by the results of a control experiment in which a *B. coli* culture was incubated for a few hours. Thereafter, when the maximal rate of growth had become established, the total numbers present being sub-maximal—the culture was exposed to a temperature of 0° C., and a diminution in the numbers was observed corresponding with that found in the previous experiment; in short, the death of the organisms was due to the effects of exposure to the lower temperature.

It is inconceivable that the effect of a lowered temperature on organisms can be summed up entirely by the expression that some die. An influence sufficiently noxious to kill the weakest must have a baneful effect on the survivors. The following experiment bears out this point.

The culture used for Experiment PPS had, thirty-seven hours previously, been subdivided, and one portion exposed to 0° C. A small portion of the refrigerated culture was then inoculated on to fresh peptone water with the following result:—

TABLE PPSF, corresponding with CURVE PPSF (CHART II. p. 25).

Age of Parent Culture.	Age of Subculture.	No. per c.c.	Log ₂ .
At 20° C. 361½ hours . . . At 0° C. 37 " . . . Total 398½ " . . .	0 hours	275,000	18·07
	5 "	272,000	18·05
	8 "	1,375,000	20·39
	11 "	7,350,000	22·81
	14 "	28,000,000	24·74

A reference to Table L shows that restraint in growth of organisms from a parent culture at 20° C. more than 75 hours old increases but slightly with advancing age, and for practical purposes the difference in restraint of growth for organisms obtained from cultures respectively, 361½ and 398½ hours old, may be neglected. Whatever difference, therefore, appearing in the restraint of growth in Table PPSF as compared with the result in the experiment represented by Table PPS, may therefore be regarded as due, entirely, to the effect of exposure to the lowered temperature—0° C. for 37 hours.

Comparing Tables and Graphs PPS and PPSF (Chart II.), the following points become evident:—For the frozen organism: absolute restraint of growth for five hours, partial restraint for 1½ hour. Measured graphically: total delay = 5½, whereas had the culture not been so exposed the growth delay = 4½. The effect of exposure for thirty-seven hours at 0° C. in this experiment may therefore be estimated as = 1;—measured in terms of the minimal period during which organisms double their numbers. By a previous experiment (Table SF) it is plain that, in addition to delay in growth of such organisms as survive the test of exposure at 0° C., others are killed in numbers which appear to increase directly as exposure is prolonged.

Again, to test the action of a preservative substance on *B. coli*, a portion of the culture used both for experiment PPS and PPSF was taken, and to it was added sterilised borax, sufficient to saturate the solution. After thirty-seven hours incubation at 20° C., subcultures on to fresh peptone water were made, and the organisms present estimated at frequent intervals. The following are the results:—

TABLE PPSB and corresponding CURVE (CHART II. p. 25).

Age of Parent Culture.	Age of Subculture.	No. present per c.c.	Log ₂ .
361½ hours . . . Borax added, 37 " . . . Total, 398½ " . . .	0 hours	60	5.9
	5 "	...	...
	8 "	60	5.9
	11 "	110	6.78
	15½ "	640	9.33
	16½ "	1,090	10.09
	29½ "	28,700,000	24.77
	35 "	42,000,000	25.32
	38 "	84,000,000	26.32
	39 "	128,000,000	26.93
	53½ "	191,250,000	27.91

There is absolute restraint of growth lasting for five hours, followed by slight growth. Later, towards the seventeenth hour, the organisms are beginning to multiply rapidly, and thereafter no restraining effect can be detected. Graphically, this delay in growth is estimated at 15, and deducting 4½ as the measure of the delay due to staleness of the medium (as for Experiment PPS), the actual effect of the presence of the borax on the organisms may be estimated as 10½; whereas, for a like period of exposure to a temperature of 0° C., the delay in growth is measured by 1.

Further experiments with borax at low concentration show that for short periods there is no alteration in numbers of the organisms present; for higher concentration large numbers are killed, the death-rate varying directly with the period of exposure to the action of the preservative.

2. Growth of *B. coli* in Dulcitate Broth.

Dulcitate, 1 per cent.; Peptone, 1 per cent.; NaCl, ½ per cent.;
Water: sterilized.

The experiments were continued at 20° C., using in place of peptone water dulcitate broth of the composition indicated. The strain of *B. coli* used for inoculation is unchanged save that, in order to eliminate as far as possible the effect of previous growth in peptone water, a series of subcultures from dulcitate broth to dulcitate broth were made; and finally, it having been determined by trial experiments that, at about the twelfth hour of incubation of any dulcitate subculture, rate of growth is maximal, the following experiment to determine period of minimised restraint of growth was carried out. The numbers of organisms present are determined according to methods used in connection with peptone water.

The following is the result:—

TABLE D, and corresponding CURVE D (CHARTS I. and III. pp. 25 and 26).

Age of Parent Culture.	Age of Subculture.	No. of <i>B. coli</i> per c.c.	Log ₂ .
14½ hours	0 hours	480	8.90
	1½ "	390	8.60
	2½ "	480	8.90
	3½ "	560	9.13
	4½ "	890	9.79
	7½ "	4,500	12.13
	9½ "	20,000	14.28

This table appears to indicate that there is a slight fall in the number of *B. coli* at the period one and three-quarter hours following inoculation. Such apparent fall might be due to clumping of the added organisms, assuming that no real reduction in numbers had taken place. In this case the apparent fall in numbers is a fallacy inseparable from the method of plating used to estimate numbers of living organisms present in any given quantity of medium. The experiment was thrice repeated, and the slight reduction appears constantly, although special efforts were made to break up such clumps, if any, by violent and prolonged agitation of the inoculated medium, hanging drops of which, examined microscopically from time to time, furnished no evidence of agglutination or aggregation of organism.

Thus, under conditions of minimised restraint of growth, with minimum change in the surrounding nutrient medium, there is a period of absolute quiescence for two and three-quarter hours; more, there is apparently a slight reduction in the numbers of organisms present. Following on this, the period of absolute quiescence, there is, for one hour, slight growth; and finally, for a period of four hours, submaximal growth.

Graphically measured, the Curve D shows that the total restraint or delay in growth or latency = 4, i.e. if the rate of growth had been maximal from the beginning the numbers of organisms present at seven and three-quarter hours would have been sixteen ($= 2^4$) times as many. In this experiment the organisms, when growing at a maximal rate, appear to double their numbers hourly.

Compared with the rate of growth in simple peptone water under comparable conditions of minimised restraint in growth, the delay with dulcete broth is somewhat remarkable. Comparing Curves D and P (Chart I.), total latency (or restraint of growth) with dulcete broth is four times as great as with peptone water, and with the latter medium there is no period of absolute quiescence or absolute latent period, nor is there observed even an apparent reduction in the number of organisms present. Curve P clearly crosses Curve D shortly after the third hour following inoculation, although in the experiment with peptone water the number of *B. coli* present at the beginning is but one-fourth the number present in that carried out with dulcete broth. Nevertheless, with peptone water, there is a definite restraint in growth. The phenomenon may be explained by the assumption, first, that organisms when growing at their maximal rate are in a state of maximal metabolism, and that correspondingly the rate of ingestion of the surrounding pabulum is maximal; finally, that, as a condition necessarily antecedent to such condition of maximal ingestion, the organism must have secreted or added to the nutrient medium within its immediate zone of activity certain substances of such a nature as will cause or fit that medium to be most readily absorbed by the protoplasm of the organism, so that if now the organisms, though multiplying at their maximal rate, be transferred to fresh nutrient medium of like composition, fresh substance must again be secreted to prepare the nutrient medium for absorption, the former ingestion-preparatory substance having become diluted and rendered comparatively infinitesimal in the process of subculture.

From a series of trial experiments it had been determined that at the one hundredth hour following inoculation of a dulcete broth medium there was no increase in the numbers of organisms present, and by the hundred and fiftieth hour the tendency was for the numbers to begin to show a slight decrease. Apparently the number of organisms present in a culture of the age one hundred and fifty hours is maximal; the fact that all the original unchanged nutrient medium has been accounted for, and the accumulated products of activity of the organisms are beginning to exercise their several effects in that increase of numbers is entirely stayed, some of the less resistant organisms are dying, while injury is being done to the survivors,

is marked by their slow recovery and delayed multiplication when placed in the most favourable conditions for growth. Inspection of the curve DD (Chart III. p. 26) shows absolute quiescence of the organisms present for five hours, for the next two and a half hours a slight increase in numbers, and for yet another six hours a submaximal rate of growth; not till the thirteenth hour following time of inoculation does growth become maximal, and by direct measurement the total restraint or delay in growth or latency is equivalent to eight hours' growth. Following previous notation, latency = 8.

Experiments to determine delay in growth due to age of the parent culture were carried out, with the following results:—

TABLE DD1 and DD2, incorporated in corresponding CURVE DD
(CHART III. p. 26).

Age of Parent Culture.	Age of Subculture.	No. of <i>B. Coli</i> per c.c.	Log ₂ .	Age of Parent Culture.	Age of Subculture.	No. of <i>B. coli</i> per c.c.	Log ₂ .
Hours 152½	Hours. 0	256,500	17·97	Hours 164½	Hours. 0	247,000	17·91
	12¾	11,250,000	23·42		2½	247,000	17·91
	15¼	40,300,000	25·26		5	267,500	18·08
	17¾	89,000,000	26·41		7½	376,000	18·52
	20¼	150,000,000	27·16		12¾	6,025,000	22·52
	25½	446,600,000	28·73		25	129,000,000	26·94
	37¾	620,000,000	29·21		30	187,000,000	27·48
	42¾	755,000,000	29·49		50½	355,000,000	28·40
	63¼	980,000,000	29·97		74¾	810,000,000	29·59
	68	1,065,000,000	29·99				

Comparing this result with Tables PP and PPS (Chart I. p. 25) wherein latency to translation of organisms from a stale medium, seventy-five and four hundred hours old respectively, is found to vary from 4 to 4½, it is clear that the prolonged period of absolute quiescence is sufficient to account for the greater value, 8. In neither PP nor PPS Curves does there appear any period of absolute quiescence; and the period of quiescence observed in Curve DD (five hours) might be predicted from the course of Curve D, wherein absolute quiescence is two and three-quarter hours; for in the latter experiment organisms were transferred when multiplying at their maximal rate to fresh medium; whereas in the former experiment the organisms transferred were in a state of stagnation, suffering perhaps from injury due to intoxication by the effete products of their own activity. Whereas PP Curve compared with P Curve showed an increase of 2½ in terms of latency, i.e. an increase of at least 250 per cent., so might there be predicted a corresponding increase of latency with parallel experiments conducted with dulcete broth in place of the peptone water, an estimate which yields a resultant greater than 7.

Lastly, to determine the action of dulcete broth on organisms taken from a stale peptone water culture, the following experiment was carried out:—

TABLES DP1 and DP2, corresponding composite CURVE DP
(CHART III. p. 26).

Age of Parent Culture.	Age of Subculture.	No. of <i>B. coli</i> per c.c.	Log ₂ .	Age of Parent Culture.	Age of Subculture.	No. of <i>B. coli</i> per c.c.	Log ₂ .
Hours 87½	Hours.			Hours 97½	Hours.		
	0	230,000	17·81		0	300,000	18·19
	2½	230,000	17·81		1	300,000	18·19
	5	251,333	17·94		13½	17,500,000	24·06
	7½	660,000	19·33		13½	51,000,000	25·60
	10½	3,100,000	21·56		23½	102,000,000	26·60
	11½	4,933,000	22·23		78	560,000,000	29·06
	24	125,000,000	26·90		125½	810,000,000	29·59
	29	190,000,000	27·50				
	34	265,000,000	27·98				
	77	800,000,000	29·57				
	124½	980,000,000	29·87				

Here the total restraint in growth is $6\frac{1}{2}$, made up of not less than two and a half hours absolute quiescence, and the remainder the equivalent of the period of submaximal growth. Compared with Curves, PP in which the period of staleness of the parent culture was almost identical and the period of restraint in growth is 4, the difference of $2\frac{1}{2}$ is that which may be almost predicated as due to that period of absolute quiescence which is associated with the transfer of organisms to dulcitate broth culture even under the most favourable conditions. (Curve D, Charts I. and III.)

REVIEW.—Thus it becomes abundantly evident that there is a definite and fixed period of incubation, or latent period for organisms when transferred to dulcitate broth, and this period is constant under all conditions. Previous injury to the added organisms has the effect of prolonging the period of incubation. The latent period is, with dulcitate broth, one essentially of absolute quiescence of the organisms present, while in some of the experiments (D) there appeared to be a slight reduction of their numbers. The period of quiescence is followed by a period of slight activity, which shortly becomes submaximal and finally maximal.

With peptone water, under like conditions of experiment, there is a period of restrained growth on incubation, which, however, is short, the total delay not exceeding one hour: the period of absolute quiescence, if present, is so brief as to elude detection under the conditions of the experiment. With previous injury to the added organisms—such as is produced by the toxic effect of the products of metabolism—the restraint in growth, now prolonged, is directly influenced by the staleness or degree of injury; it varies somewhat with the age of the stale organism. Exposure of the organisms to low temperatures for prolonged periods kills many, and of necessity must have a deleterious effect on some of the survivors. Such effect becomes visible and can be gauged precisely in terms of restraint of growth of the organism when transferred to a fresh nutrient medium under normal conditions of activity and growth.

On organisms the action of a preservative, such as borax, is variable. With a slight degree of concentration there may be an arrest of growth; with an increasing amount the number of organisms present begins to diminish: the least resistant are killed, the less resistant maimed. The number and rate of killing may be measured directly by the method of plating; the amount

and rate of maiming by the delay in growth of the organisms present when placed in most favourable conditions for repair and recovery (Curve PPSB).

3. Growth of *B. coli* in Milk.

(a) Fresh goat's milk was sterilised by heating to 100° C. on three successive days, and inoculated subsequently with *B. coli* derived from a culture in boiled milk. Temperature of experiment throughout = 20° C. Results as follow:—

TABLE and CURVE bb (CHART IV. p. 26).

Age of Parent Culture.	Age of Subculture.	No. of <i>B. Coli</i> per c.c.	Log ₂ .
18 hours	0 hours	450,000	18·78
	1½ "	500,000	18·93
	3 "	600,000	19·19
	5½ "	950,000	19·85
	10 "	5,000,000	22·25
	24 "	530,000,000	28·98
	50½ "	750,000,000	29·48
	74½ "	850,000,000 (coagulation)	29·66

Inspection of the figure bb shows almost absolute restraint of growth (=latent period) for one and a half hours, and a gradual acceleration of growth thereafter till the sixth hour, when growth becomes maximal. Measured graphically, the total measure of restraint or latency corresponds with four hours' maximal growth, the following being the factors:—latent period, absolute delay = nearly one and a half hours; comparative delay = above two and a half hours. No diminution in numbers is observed throughout the experiment.

(b) Experiment (a) is repeated, save that at the eighteenth hour of growth the parent culture is placed on ice for twelve hours; thereafter boiled milk is reinoculated. Results as follow:—

TABLE and FIGURE bbF (CHART IV. p. 26).

Age of Parent Culture.	Age of Subculture.	No. of <i>B. Coli</i> per c.c.	Log ₂ .
18 hours; placed there- after on ice for 12 hours	0 hours	440	8·78
	5 "	440	8·78
	6 "	560	9·13
	11 "	4,150	12·019
	22½ "	3,000,000	21·51
	24 "	5,000,000	22·25
	30 "	80,000,000	26·25
	48 "	750,000,000	29·48
	72 "	770,000,000 (coagulation)	29·52

Inspection of figure bbF shows absolute delay of growth for five hours; at the expiration of this period, sudden growth occurs with maximal acceleration, and at hour ten and two-thirds growth is maximal. Total latency or restraint=seven hours' growth; latent period=5; comparative restraint=2.

Thus, comparing results of experiments bb and bbF, the total added restraint in growth due to freezing of *B. coli* for twelve hours=three hours; the period of absolute quiescence in the early experiment being increased by one and a half hours, and the period of comparative quiescence being prolonged by the equivalent of one and a half hours.

Comparing the results of this experiment with that of PPSF, wherein stale peptone water culture is exposed to 0° C. for thirty-seven hours with an added delay due to freezing of only one hour, it might appear that actively growing organisms are susceptible to the harmful influence of low temperatures to an unusual extent. The series of experiments bb and bbF, PPS and PPSF are not, however, strictly comparable. The former pair shows the effect of freezing on actively growing organisms; the latter, on apparently inactive, injured, and dying organisms. The noxious influence of exposure to low temperatures in the former would appear to be at a maximum; in the latter at a minimum. The varying results are almost comparable to those which obtain when healthy and sickly small laboratory animals are exposed respectively to the influence of a noxious substance, such as coal gas; death with the former usually occurs rapidly; with the latter there is variable delay. With the healthy organisms the low temperature may operate in the direction of minimised metabolic activity in general, and minimised absorption in particular; with stale, inactive, injured, and dying organisms, with such a metabolism that the process of absorption is equivalent almost to the process of excretion, the staying of the process of noxious absorption is in effect an arrest of the injury, and if, as may be, the process of excretion is stayed to a less extent, then the whole process of exposure to a low temperature means, in effect, an opportunity for the recovery from the previous harmful influence. The organism now is starved; no longer poisoned. This effect is seen plainly in Experiment SF.

Nevertheless, freezing of itself has an injurious effect, as is plain enough from the column of figures showing the number of organisms surviving at given dates; many are killed, some of the survivors must be injured.

Freezing, while staying in general the vital activity of an organism, would seem to have a twofold influence: metabolism is minimised; the lowered temperature is directly injurious to the organisms. The stay of metabolism when the metabolic process is of itself injurious has a beneficial effect; the lowered temperature would appear to be injurious directly in proportion to the time factor. The previous state of activity of the organisms is, however, important. It may be that freezing, while the equivalent of starvation in its stay of absorption, may be, in an extremely active organism, the equivalent of a noxious effect in that a process of auto-intoxication is set up due to the stay in excretion.

Of the stay in absorption at lowered temperatures there is little doubt; the experiment of exposure of an active organism to a germicide at appropriate temperatures is the direct proof; other conditions remaining constant, the germicidal rate is rapidly reduced directly with the lowering of the temperature.

(c) *New milk*.—Goat's milk is collected with ordinary aseptic care, cooled immediately to 20° C., and, without delay, inoculated from a new-milk culture of *B. coli*, age twenty-four hours,—at which period previous trial experiments had shown rate of multiplication of *B. coli* to be maximal.

Rate of growth is as follows :—

TABLE and FIGURE NN (CHART IV. p. 26).

Age of Parent Culture.	Age of Sub-culture, i.e. from time of Milking.	No. of <i>B. coli</i> per c.c.	Log ₂ .
24 hours	0 hours	584,000	19·15
	6 „	565,000	19·10
	10½ „	1,110,000	20·08
	24½ „	2,530,000	21·28
	50½ „	167,000,000	27·31
	...	(coagulation)	...

From this it is plain that there is no growth during hours 0 to 6 following milking; moreover, there is undoubted reduction in numbers; thereafter growth takes place, slight at first, later increasing rapidly towards the twenty-fourth hour, when growth becomes maximal.

Comparing the rate of growth of *B. coli* in boiled and new milk respectively by means of bb and NN curves, it is clear that one or more factors absent in boiled milk dominate the growth in new milk. Some explanation might be sought from the fact that the two experiments are not strictly comparable; the parent cultures are different. Direct experiment, however, by transferring organisms from a parent boiled-milk culture of *B. coli* to new milk, show results in growth not differing appreciably from those obtained in NN Experiment.

There is a significant crossing of the two curves bb and NN as early as the third hour after the commencement of the experiment; the rate of growth in boiled milk rapidly makes up for difference in numbers of organisms originally added. Further, at no period of growth of *B. coli* in new milk does the rate of increase appear to equal the maximal rate observed in boiled milk.

Measured graphically, the total delay in growth for new milk is equal to ten hours maximal growth; absolute inhibition and delay coupled with some reduction in numbers account for six hours, partial inhibition accounting for the remainder. The diminution in number during the first six hours is a phenomenon which occurs constantly in all experiments with *B. coli* in new milk. Clumping of the organisms might be held to account in some measure for the phenomenon, but this is negatived by the fact that continuous violent agitation of the medium immediately before counting takes place has no appreciable effect in increasing the estimate of the number of organisms present.

The delay in growth in boiled milk with minimised latency was found to be equivalent to a total of four hours delay, and the superadded factor of latency for new milk must therefore be the equivalent of six hours' growth.

Preservative Substances.—The effect of adding boric acid and borax respectively to new milk samples immediately following milking, as a preliminary to inoculation with *B. coli*, showed results which threw some light

on the phenomenon, already noted, of reduction of numbers of *B. coli* in new milk during the first six hours. In these experiments, adding from $\frac{1}{2}$ to 2 per cent. of preservative to the milk, no increase in numbers of *B. coli* was noted. On the other hand, *there was no reduction in numbers*, a fact which may be explained on the assumption that there is present in new milk some factor which not only inhibits growth of organisms for some time, but in addition destroys a certain number. This factor is held in check by the action of the preservative; there is in effect a "stay of execution." On the other hand, the action of the preservative substance as affecting growth in new milk is precisely similar to that observed for boiled milk,—with like quantities growth ceases. The reduction in numbers observed then, at the commencement of Experiment NN, is not due to clumping of the organisms; the explanation seems well founded that in new milk there is some factor at work, which, with the organisms under review, has the effect of destroying many of those present, and effectively staying the growth of the survivors during hours 1 to 6, while subsequently there is a comparative restraint in growth which finally passes away at the twenty-fourth hour. The total measure of restraint during hours 6 to 24 is alone precisely equivalent to the total measure of restraint as observed for boiled milk from the commencement of the Experiment bb; and the superiority of new milk over boiled milk as a medium for checking the growth of *B. coli* is thus shown to be equivalent to six hours' maximal growth, which may be taken as a measure of the value of the bactericido-inhibitory power of new milk, apart from the period of latency or restraint in growth equal to four hours' maximal growth possessed by new milk in common with boiled milk, as a factor of minimised latency, or period of incubation; the latter may be defined as the minimum period required by an organism to regain its normal maximal rate of growth when transferred rapidly growing from one nutrient medium to another of like composition.

Refrigeration.—The effect of reducing the temperature of new milk for twenty-four hours to 0° C. immediately following milking, and thereafter inoculating the new milk now raised to 20° C. with *B. coli* from a twenty-four hours' old new-milk culture, as with Experiment NN, shows the persistence of a period of absolute inhibition with slight bactericidal power, for three hours, followed by a partial restraint of growth for a further nine hours, the equivalent of a total of seven hours' restraint of maximal growth, of which that of the last four differs in quantity in no wise from that observed with NN Experiment. A second experiment carried out under almost identical conditions, save that the new milk had been reduced to 0° C. for forty-eight hours before inoculation, showed subsequently that the period of absolute inhibition had almost disappeared; the total measurement of restraint of growth was slightly in excess of that which obtained for boiled milk, as in bb Experiment.

Thus the effect of refrigeration for forty-eight hours is to abolish, almost completely, the advantage of new milk (i.e. its bactericido-inhibitory power), as compared with boiled milk, in restraining the growth of *B. coli*.

Blood heat.—The effect of growing *B. coli* in new milk and boiled milk respectively at blood heat—the organisms being derived from the several like parent cultures incubated at 37° C.—shows that with new milk the bactericido-inhibitory period persists for $1\frac{1}{2}$ hour only, followed by some restraint of growth during the following $4\frac{2}{3}$ hours. At the sixth hour the growth becomes maximal; while with boiled milk there is immediate increase of numbers from the commencement of the experiment. The advantage of new milk over boiled milk measured graphically is $1\frac{2}{3}$ hour maximal growth, being slightly in excess of the equivalent of the period of bactericido-inhibitory action of the new milk. In effect the special bactericido-inhibitory quality of the milk wanes rapidly at 37° C., its presence being no longer detected after the lapse of two hours.

TABLE showing Rate of Growth of *B. coli* in Milk.

Hour.	Numbers present per $\frac{1}{100}$ c.c.								
	0	$\frac{1}{2}$	1	$1\frac{1}{2}$	2	3	4	5	6
New milk .	92	91	88	113	153	920	3,200	10,000	40,000
Boiled ,, .	92	112	131	162	250	1250	14,400	120,000	>500,000

REVIEW.—New milk possesses special qualities, one of the effects of which is to destroy a certain number of added organisms. In addition there is a definite inhibition or arrest of growth of the survivors for a period dependent on the conditions of exposure of the milk subsequent to milking. At 20° C. this quality of absolute inhibition persists for six hours, and at 37° C. for rather more than one hour. If the temperature of the milk be raised to 100° C. momentarily the special inhibitory quality of the milk entirely disappears. If the milk be kept at 0° C. the special quality can be shown to persist at the end of twenty-four hours to one-half of its initial extent provided now the milk be raised to 20° C.; while at the end of forty-eight hours under like conditions but a mere trace of the bactericido-inhibitory power persists.

The action of the preservatives used is to destroy the bactericidal power of the milk, and if it be granted that a property which has the effect of destroying some organisms necessarily restrains or inhibits the growth of the survivors, then the effect of the preservative must be regarded as destructive of the bactericido-inhibitory property of new milk.

A quality, waning but slowly with the lapse of time if the milk be kept at freezing-point, lasting for several hours at summer heat, and for one hour only at blood heat, capable of immediate destruction by heating to the boiling-point or by the addition of chemical substances such as preservatives, cannot be regarded either as physical or chemical. It must be due to some property of new milk specially associated with its origin from living tissue; it cannot be other than a vital or physiological phenomenon.

III. GROWTH OF VARIOUS ORGANISMS IN NEW MILK.

Contrasting the growth of *B. coli* in boiled and new milk respectively as carried out in NN, bb, and bbF experiments, certain points become evident.

1. The maximal rate of growth of *B. coli* in new milk never reaches that for boiled milk.

2. The total numbers of *B. coli* in new milk at the end of forty-eight hours' incubation are 160 millions per c.c., while for boiled milk the numbers are 750 millions; thereafter the proportion does not vary materially so long as clotting is absent.

To elicit facts relative to these points, new milk was inoculated as in Experiment NN, and thereafter kept at 20° C. The numbers of the various classes of organisms present were then estimated at regular intervals by methods of plating on agar at blood heat and gelatin

at 20° C. Organisms present other than *B. coli* were estimated by methods of differences; the following tables show the results:—

TABLE NN2 (CHART V. p. 27).

Hours subsequent to milking.	<i>B. coli</i> (=C). Taurocholate Agar Plates at 37° C.	<i>Non-coli.</i>		Total flourishing—	
		Agar Plates at 37° C. (=B).	Gelatin Plates at 20° C. (=G).	At 37° C.	At 20° C.
At milking) time	363	1,037	177	1,400	540
2	275	1,125	225	1,400	500
4	198	1,202	602	1,400	800
6	385	2,135	915	2,520	1,300
9	3,080	4,920	10,520	8,000	13,600
12	23,100	...	(?) 6,900	23,100	(?) 30,000
20	110,000	...	190,000	110,000	300,000
22	220,000	...	(?) 180,000	220,000	(?) 400,000
24	660,000	...	500,000	660,000	1,160,000
30	11,000,000	...	4,000,000	11,000,000	15,000,000
36	121,000,000	...	79,000,000	121,000,000	200,000,000
48	440,000,000	...	(?) 10,000,000	440,000,000	450,000 000
		First sign of clotting			
60	440,000,000	...	60,000,000	440,000,000	500,000,000

TABLE NN2 (CHART V. p. 27), Log₂.

Hour.	<i>B. coli</i> Taurocholate Agar Plates at 37° C. (C).	<i>Non-coli.</i>		Total.	
		Agar Plates at 37° C. (B).	Gelatin Plates at 20° C. (G).	At 37° C.	At 20° C.
0	8·50	10·01	7·46	10·45	9·07
2	8·10	10·10	7·81	10·45	8·96
4	7·63	10·23	9·23	10·45	9·64
6	8·589	11·06	9·83	11·30	10·34
9	11·59	12·26	13·36	12·96	13·73
12	14·49	...	(?) 12·75	14·49	(?) 14·87
20	16·74	...	17·53	16·74	18·19
22	17·74	...	(?) 17·45	17·74	(?) 18·61
24	19·33	...	18·93	19·33	20·07
30	23·39	...	21·93	23·39	23·84
36	26·85	...	26·23	26·85	27·57
48	28·71	...	(?) 23·25	28·71	28·74
		First sign of clotting			
60	28·71	...	...	28·71	28·90

The organisms present in the milk are of several varieties.

(a) Those added, *B. coli*, capable of flourishing both at 37° C. and at 20° C.

(b) Those derived from the milk ducts, having there obtained a

nidus by contamination from the skin or from the mouth of the sucking kid, or from other external source. They flourish at 37° C., and when transplanted to agar plates at a like temperature form visible colonies overnight; they do not flourish at 20° C.

(c) Those derived from sources as for (b). They flourish at 20° C., and when transplanted to gelatin plates at 20° C. form visible colonies after three days' growth.

The growth of these several varieties is as follows:—

(a) Hours, 0 to 4—reduction in numbers by 45·4 per cent., which indeed may be taken as the estimate for the bactericidal power of the new milk. For hours 4 to 6 there is a recovery in the numbers to those originally present; thereafter growth continues.

(b) Hours, 0 to 4—steady increase in numbers; thereafter, hours 4 to 9, a steady acceleration of growth. After the ninth hour the organisms present, if any, do not admit of estimation by means of the method of differences. These organisms may be presumed to have suffered one of two fates.

(i) Assuming their normal nidus to be the skin or milk-ducts with a temperature of 37° C., then their early multiplication in the milk at 20° C. outside the body may be due to the removal or waning of the inhibitory power of the milk. Later, the continued exposure to a lowered temperature of 20° C. is followed by an attenuation of the organism, as marked by their falling numbers; or—

(ii) Their multiplication is stayed to such an extent by the rapidly accumulating products of growth of *B. coli* and other organisms that their numbers become comparatively insignificant, even if they do not actually dwindle.

(c) These organisms show a rapid growth from the beginning. For hours 0 to 2 there is an increase of 25 per cent., and for hours 2 to 4 the numbers are, at least, doubled; thereafter there is a steady increase.

[The observations at the twelfth, twenty-second, and forty-eighth hours would appear to be experimental errors, due probably to inaccurate graduation of measuring pipettes in use.]

REVIEW.—The difference as to total numbers of *B. coli* present in boiled and new milk experiments previously quoted may be accounted for by the presence in the new milk of large quantities of organisms other than *B. coli*. These flourish, especially at 20° C., both in the new milk and subsequently when transplanted on to gelatin plates at like temperature. Other organisms present in new milk flourishing at 37° C., do not appear, in the condition of the experiment, to have any retarding influence.

Those organisms present in milk and derived from the milk ducts, whatever their prime source, must necessarily be such as have already survived or resisted the bactericidal action of the milk up to the time of milking. The absence of any subsequent decrease in their numbers does not necessarily imply that the milk at no time possessed special

bactericidal powers towards them; but in any case the absence of absolute inhibitory phenomena may be significant of the fact that the bactericidal power of the milk, specially towards them, is scanty or limited, although on the other hand their comparative paucity in the milk might be taken as evidence of a strong inhibitory and perhaps bactericidal power of the milk while contained within the milk ducts. Even if there be no absolute inhibitory power of new milk towards such organisms, there is a marked difference in their comparative restraint of growth in milk outside the body. For such organisms as flourish at blood heat, during the first six hours the organisms are scarcely doubled in numbers, while during the first two hours the organisms increase only by about 8 per cent.; on the other hand, for organisms which flourish at air temperature, and in all probability are derived from sources entirely alien to the animal tissues, the growth during like periods is threefold as great.

The broad fact remains established, that in new milk there are organisms present which have a modifying influence on the growth of those added; and further, the bactericido-inhibitory action of new milk is a factor varying widely with the class of organism present or added.

IV. MASS ACTION.

By this term is indicated the manner of growth in a medium of a mixture of organisms the several varieties of which appear to be capable of free growth when existing independently in that medium. In the experiments hereunder referred to, the factor of delay in growth due to latency has been minimised by inoculating the several test media with organisms derived from like media in which free growth is already taking place. The parent culture is eighteen hours old. The test temperature for ascertaining rate of growth in the various media throughout these experiments is 20° C.

The numbers given are the average of at least six plates.

EXPERIMENT M1.—*New Milk*.—Taking the number of *B. coli* appearing on the neutral-red taurocholate-lactose-agar plates, and comparing with the total number found on the gelatin plates incubated at 20° C., the results are as follow: If there be an approximation of the numbers in the beginning, after the first three hours, there is a disturbance of the ratio in favour of the organisms appearing on the gelatin plates—due to the waning of the absolute-inhibition effect of the new milk for organisms other than *B. coli* which flourish at air temperature.

This disturbance is in favour of the gelatin plates till the sixth hour, when the inhibitory effect of the new milk on *B. coli* passes off. Thereafter up to the thirty-sixth hour the numbers on the taurocholate plates tend to approximate those on the gelatin plate, so that by the time clotting commences the ratio of *B. coli* to other organisms which flourish at air temperature is little changed from that which held at the beginning of the experiment. Organisms other than *B. coli* which flourish at blood heat appear to be obliterated after the sixth hour, and do not appear on the estimating plates in high dilution.

TABLE M1.—*New Milk inoculated with B. coli ex New Milk Culture.*

Hour.	<i>B. coli</i> Colonies. Taurocholate Agar Plates.	Non-coli Colonies. Agar at 37° C.	Gelatin at 20° C. Coli + Non-coli Colonies.	Ratio of Gelatin to Taurocholate Agar Colonies.
0	363	1037	540	1·48
2	275	1125	500	1·81
4	198	1202	800	4·0
6	385	2135	1,300	3·3
20	110,000	...	300,000	2·7
24	660,000	...	1,100,000	1·66
30	11,000,000	...	15,000,000	1·36
36	121,000,000	...	200,000,000	1·65
48	440 000,000	...	450,000,000	1·02
Clotting commencing				

EXPERIMENT M2.—*New Milk.*—The ratio of organisms appearing on the gelatin plates, compared with those on the taurocholate plates, is greater: 9·2/1. After the fifth hour there is an increasing disparity, which appears to become maximal (31/1) about the thirtieth hour. The ratio of *B. coli* to other organisms which appear to be capable of flourishing at blood heat appears to be maintained throughout.

TABLE M2.—*New Milk inoculated with B. coli ex New Milk Culture.*

Hours.	<i>B. coli</i> Colonies. Taurocholate Agar Plates.	Agar.	Gelatin.	Ratio of Gelatin to Taurocholate Agar Colonies.
2	390	2,150	3,650	9·2
4	390	...	...	...
6	500	...	...	...
8	920	4,000	10,000	10·9
26½	4,250,000	9,520,000	136,000,000	31
50½	39,000,000	195,000,000	1,110,000,000	28·5

EXPERIMENT M3S.—*Sterilised milk* inoculated from a culture of organisms in sterilised milk. These organisms are derived originally from natural milk to which *B. coli* has been added. The ratio of *B. coli* to those appearing on the gelatin plates was practically identical with that of Experiment M2. Inhibition as a factor is entirely absent, and latency is minimised; there is an increased growth for all organisms from the beginning,

and the ratio is likewise disturbed. In the beginning the ratio is 9·8/1; it increases gradually to the twenty-fifth hour, when it reaches 48/1; this disparity increases to 262/1 towards the fiftieth hour, when clotting commences. Organisms other than *B. coli* which flourish on agar at blood heat appear in the beginning to increase at a rate approximating those on the gelatin plates, but after the seventh hour the rate appears to be diminished to an extent greater than for *B. coli*.

TABLE M3S.—*Sterilised Milk.*

Hours.	<i>B. coli</i> Colonies. Taurocholate Agar Plates.	Agar.	Gelatin.	Ratio of Gelatin to Taurocholate Agar Colonies.
1	580	800	5,700	9·8
5	680	1,900	9,400	14
7	1,440	8,300	33,600	23·3*
25	7,300,000	7,800,000	350,000,000	47·9*
49½	6,300,000	7,500,000	1,750,000,000	262*

* Ratio practically = *B. lactici acidi* to *B. coli*.

EXPERIMENT M4—*New milk* is inoculated from a new milk culture of organisms naturally found in milk, with in addition *B. coli*. The preponderating majority of those organisms, non-coli, which grow at 20° C. in this case, are lactic acid bacilli (12,000 out of 12,500). The ratio of non-coli to coli on gelatin plates in the beginning is 480/1. This ratio rapidly increases, and at the twenty-fourth hour is 4166/1; then, to the time of clotting

TABLE M4.—*New Milk.*

Hours.	<i>B. coli</i> Colonies. Taurocholate Agar Plates.	Agar.	Gelatin.		Ratio of <i>B. acidi lactici</i> to <i>B. coli</i> .
			Total.	Lactic acid bacilli.	
1	25	290	12,500	12,000	480
4	30	430	...	...	...
7	20	570	...	...	...
24½	6,000	...	25,000,000		4,166
30½	26,600	...	97,000,000		3,650
48	510,000	...	1,600,000,000		3,137

at the forty-eighth hour, there is a slight fall. There is an overwhelming ratio of lactic acid bacilli to other organisms which flourish at air temperature on gelatin plates: the latter are difficult to detect and estimate. Non-coli organisms flourishing at blood heat on agar are difficult to detect or estimate after the seventh hour.

EXPERIMENT M5.—In this the milk used is new milk which, since milking, has been standing on ice for twenty-four hours. Estimation of *B. coli* shows that the absolutely inhibitory period now only persists for three hours, i.e. one-half of the period for milk quite new, while for lactic acid organisms the period is correspondingly diminished below three hours. The ratio of lactic acid bacilli to *B. coli*, 3000/1 in the beginning, increases to 28,500/1 at the end of twenty-four hours, the growth of *B. coli* being vastly restrained throughout. At the forty-ninth hour the ratio is increased to < 333,000/1, i.e. more than a hundredfold.

TABLE M5.—*Partial Loss of Inhibition. New Milk put on Ice for Twenty-four Hours before Inoculation.*

Hours.	<i>B. coli</i> .	Lactic Acid Bacilli.	Ratio of Lactic Acid Bacilli to <i>B. coli</i> .
0	10	30,000	3,000
3	10	36,000	3,600
6	20	80,000	4,000
24½	350	10,000,000	28,500
30	400	50,000,000	125,000
49	<3000	1,000,000,000	> 333,000

EXPERIMENT M6.—As for Experiment M5, save that the new milk has been placed on ice for forty-eight hours subsequent to milking. The inhibitory effect of the new milk for *B. coli* must persist somewhat; and this applies in a similar degree to the lactic acid bacillus. At the end of five hours there is but a slight increase, seeing that latency has been minimised. The ratio of lactic acid bacilli to *B. coli* in the beginning is 26,666/1. At the fifth hour this has increased to 40,000/1, and at the twenty-seventh hour the disparity has reached the vast proportion of < 20,000,000/1. Thus the ratio has increased about a thousandfold.

TABLE M6.—*New Milk kept on Ice for Forty-eight Hours before Inoculation. Great Loss of Inhibition.*

Hour.	<i>B. coli</i> .	Lactic Acid Bacilli.	Ratio of Lactic Acid Bacilli to <i>B. coli</i> .
0	15	400,000	26,666
5	20	800,000	40,000
27	450	> 1,000,000,000	> 20,000,000

EXPERIMENT M7.—New milk is put on ice for four days, and then inoculated as in experiments M5 and M6. The inhibitory power would appear to be nil, judging from the increase of *B. coli* in the first two hours. The ratio of *B. acidi lactici* to *B. coli* is increased tenfold by the fifty-second hour. The diminished number of *B. coli* after the twenty-sixth hour may be an experimental error, for the medium had already begun to clot, and similarly with regard to the estimation for *B. acidi lactici*. On the other hand, the high initial doses of both *B. coli* and *B. acidi lactici* added, have contributed to masking their several effects; for after a comparatively few hours, with ordinary increase, the numbers cannot be far short of maximal. Each kind of organism, for a few hours at any rate, maintains its independent rate of increase in a medium in which there happen to exist other organisms. When, however, the several effects of the products of vitality of the various classes of organisms begin to taint the fluid, the alteration of growth in the *B. coli* and non-coli organisms respectively becomes marked; but the maximal effect of this cannot be shown by a mere estimate of the numbers present at the end of the experiment, for a considerable proportion of these are already present before the maximal effect has been produced.

EXPERIMENT M7.—*New Milk put on Ice for Four Days before Inoculation.*
Inhibition negligible.

Hours.	<i>B. coli.</i>	<i>B. acidi lactici.</i>	Ratio of <i>B. acidi lactici</i> to <i>B. coli.</i>
0	180	1,500,000	8,333
2	230	...	...
26	100,000	> 1,000,000,000	> 10,000
52	30,000	2,500,000,000	83,333

REVIEW.—*Ratio of Organisms capable of flourishing at 20° C. on Gelatin*
to B. coli at varying Intervals in foregoing Experiments.

Experiment.	M1. New Milk.	M2. New Milk.	M3S. Sterilised Milk.	M4. New Milk.	M5. New Milk Frozen, 24 hours.	M6. New Milk Frozen, 48 hours.	M7. New Milk Frozen, 96 hours.
0 hours .	1·48	9·2	9·8	480	3,000	26,666	8,333
25 „ .	1·66	31	47·9	4166	28,500	> 20,000,000	> 10,000
48 „ .	1·02	28·5	262	3177	> 333,000	...	8,333
Inhibition .	Normal	Normal	Nil	Normal	Partial	Slight	Nil

When the numbers of *B. coli* added to perfectly fresh goat's milk approximate those of non-coli organisms capable of flourishing on gelatin at 20° C. the ratio is maintained throughout the experiment up to the

time of clotting. Such disparity as appears about the fourth hour is explicable on the grounds of variability in the inhibitory action of new milk.

When, however, this approximation in numbers is disturbed either by diminishing the number of *B. coli* added or by increasing the number of other organisms capable of flourishing on gelatin at 20° C., more especially *B. acidi lactici*, the disparity in the ratio towards the end of the experiment at the commencement of clotting becomes manifold, the resulting increase being geometrical rather than arithmetical.

Experiment.	Milk incubated at 20° C., subsequent to Inoculation.	Ratio in beginning of <i>B. coli</i> to Organisms growing at 20° C. on Gelatin.	Disturbance of Ratio towards end of Fermentation.
M1	New	Approximating 1/1	Little
M2	„	9/1	Increase 3-fold
M4	„	500/1	„ 9-fold
M5	New Frozen, twenty-four hours (Inhibition, $\frac{1}{2}$)	3,000/1	„ 100-fold
M6	New Frozen, forty-eight hours (Inhibition, slight)	25,000/1	„ 1000-fold
M7	New Frozen, ninety-six hours (Inhibition, nil)	8,333/1	„ 10-fold
M3S	Sterilised milk	9/1	„ 40-fold

With new milk the diminution of the ratio, after the twenty-fourth hour, in the 3 experiments, M1, M2, and M4, may be explained by the complete evanescence of the inhibitory quality of the milk after that hour, especially towards *B. coli*, and perhaps by the restraining action of the products of growth of *B. coli* on other organisms.

Such diminution does not occur with sterilised milk (Experiment M3S), nor with new milk, the inhibitory action of which has been diminished seriously by continued refrigeration at 0° C.

With regard to Experiment M7, the comparatively low disturbance of the initial ratio may be explained by the large additions of organisms in the beginning.

M2 and M3S, with practically identical ratios in the beginning, show markedly the effect of inhibition of new milk, specially towards *B. coli*, as compared with non-*coli* organisms.

With M5 and M6 it is not possible to be certain of the exact

numbers of *B. acidi lactici*, owing to the commencement of clotting. The numbers, however, are fairly approximate, as shown by control experiments.

Organisms other than *B. coli* capable of flourishing on agar at blood heat appear to obey laws similar to those governing the growth of *B. coli*. The proof of this is plain for the first seven hours of growth. Subsequently, if the numbers be small there is great difficulty in their detection and estimation; their rate of growth does not approximate that for *B. coli*. With larger numbers growth in the later stages appears to be governed by factors similar in kind, though not in degree, to those influencing the growth of *B. coli*—latency and inhibition. This marked difference may be accounted for by the fact that in new milk there is present, of necessity, a large number of organisms derived from the milk ducts of the lactating animal. The mere presence of such living organisms is proof of the failure of the bactericido-inhibitory power of the milk specially towards them, a factor which should tell in their favour when this power of the milk wanes. On the other hand, the temperature for the conduct of these experiments has been 20° C., while the optimum temperature for these organisms might naturally be supposed to be that of the body temperature of the animal from which the organisms are derived.

Yet again, these organisms derived from the animal-body have, of necessity, during the period of refrigeration, been exposed to conditions, which, in the case of *B. coli*, have led, ultimately, to a reduction in number, while their period of latency, or incubation period, when transferred to fresh media, has been materially increased.

Thus the process of freezing new milk, preliminary to inoculation and incubation at summer heat, while destroying the inhibitory quality of the milk, likewise destroys or impairs the growing power of these particular organisms; two counteracting factors, the effect of which upon their growth estimated by direct experiment.

SUMMARY.—See REVIEWS: pp. 10, 15, 17, and 22.

EXPLANATION OF CHARTS.

Ordinates=Log. to the base 2 of numbers of organisms per c.c. Abscissæ=Hour of incubation.

The general explanation of the lettering is as follows:—

P=peptone water culture.	D=dulcitate broth culture.
N=new-milk culture.	b=boiled milk culture.
B=factor of introduction of borax or boric acid to parent culture.	
S=staleness of parent culture.	
F=factor of exposure of parent culture to 0° C.	

The combination of letters, e.g. NN, means a new-milk subculture of organisms derived from new milk parent culture.

CHART V.—See Table NN2, p. 16.

- G=organisms flourishing at 20° C. on gelatin.
 B=organisms flourishing at 37° C. on agar.
 C=*B. coli*.