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ENZYMATICALLY AND PHYSICALLY INDUCED INHERITANCE CHANGES IN BACILLUS SUBTILIS

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ENZYMATICALLY AND PHYSICALLY INDUCED INHERITANCE CHANGES IN *BACILLUS SUBTILIS*

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Otto E. Landman

Sidney Halle

Medical Bacteriology Division
DIRECTOR OF BIOLOGICAL RESEARCH

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FOREWORD

Portions of this study were presented at the Eighth International Congress for Microbiology, Montreal, August 22, 1962.[11]

The technical assistance of Mr. Rudolph Knott and Mr. Don Brutscher is acknowledged with appreciation. We also thank Dr. Irving Miller for permission to use techniques for commitment developed by him.

ABSTRACT

When a culture of Bacillus subtilis strain 168 is treated with a solution of crystalline lysozyme, the bacteria pass through an osmotically sensitive bacillary phase to the protoplast stage. All of the protoplasts can be recovered as L-colony-forming elements on complex or chemically defined osmotically stabilized agar media. Protoplasts or L-bodies can be propagated in the L-state indefinitely or can be mass-reverted to the bacillary state by altering medium ingredients. In particular, several D-amino acids and soft agar inhibit reversion to the bacillary state, while hard agar and 30 per cent gelatin media induce reversion. Reversion in 30 per cent gelatin is often 100 per cent efficient and begins within five hours after plating. Penicillin-induced L-forms of B. subtilis differ from the bacillary form in their resistance to penicillin and seem to be identical to the lysozyme-induced L-forms. Their induction and resistance behavior constitutes an instance of Lamarckian inheritance. The conversion of bacteria to the L-state is attributed to the loss of a primer for cell-wall formation and cell division, while reversion is thought to be due to reinitiation of this primer. The primer is probably associated with the cell membrane. Its possible nature and parallels between the present inheritance system and previously described systems are discussed.

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I. INTRODUCTION

Experiments with penicillin-induced reverting and stable L-forms of Salmonella, Proteus, and E. coli have shown that a cellular mechanism that controls both septation and cell wall formation is inhibited in the reverting L-forms and is heritably lost from the cells in stable L-forms.[1-3] The heritable loss becomes evident when, upon withdrawal of the penicillin, the L-forms fail to revert to the bacillary form but instead continue to propagate in the L-state. This induced heritable change is clearly not a gene mutation, since all of the surviving clones have suffered the same alteration and since 50 per cent of the cells in a Salmonella population,[3] 20 per cent of an E. coli population,* and 1.5 per cent of a Proteus population[4] have been shown to be converted.

Conversion of a cell from a bacillary-colony-forming-element to an L-colony-forming-element (commitment) can occur in liquid penicillin media in the absence of cell division.[3] Once commitment has occurred, the L-bodies in suspension may continue to increase in size, but not in number: multiplication of the stable L-forms requires soft agar. It has been postulated that the L-forms require soft agar for multiplication because they have lost their ability to form septa.[1,5] The soft agar serves as an artificial division substratum by allowing the protoplasm to ooze between and subdivide itself among the agar fibers. This substitute division-mechanism by soft agar permits the cells to dispense with substances required for septation. Thus, we have shown recently that a streptomycin-dependent strain of Salmonella paratyphi that requires streptomycin for septation can get along on five per cent of its normal streptomycin requirement in the L-state on soft agar when its normal septation mechanism is not operating.[6]

Early in the study of lysozyme-induced protoplasts of Gram-positive bacteria, it was noted that these forms were unable to divide[7] even under conditions that permitted complex biological activities such as induced enzyme formation[8] and phage synthesis.[9,10] In the light of our experiments on the penicillin-induced heritable loss of a division-and-wall-synthesizing system, it seemed likely that the same system had been lost through lysozyme treatment in the process of protoplast formation. Accordingly, if one were to supply the proper hypertonic soft agar medium to lysozyme protoplasts, these bodies should give rise to L-colonies by the substitute agar division mechanism. The realization of this expectation is recounted in this publication.[11] Experiments describing the environmentally induced reversion of these lysozyme L-forms to the bacillary state are also included.

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* Landman; unpublished results.

II. TERMINOLOGY

Certain terms used in this publication are employed by different authors with broader or with narrower connotations. The sense in which these terms are used here is given below.

Inheritance: The transmission of a trait from an individual to its progeny through successive generations. Inheritance need not involve nucleic acid;[12,13] whether nucleic acid is directly involved in the present inheritance system is not known.

Stable L-forms: There are two types of stable L-forms, those that originate through gene mutation,[5,14] and those that are induced by a mass conversion process,* but continue to multiply in the L-state for successive generations after the inducing agent has been withdrawn. Mass-conversion stable L-forms vary widely in their tendency to give rise to bacillary revertants: For example, those of E. coli have never been observed to revert;* those of Proteus mirabilis revert freely.[15]

Commitment: The point in time at which a cell loses its ability to give rise to a bacillary colony and gains its capacity to give rise to an L-colony. Lysozyme-induced commitment in B. subtilis seems to coincide with the appearance of protoplast morphology.

Protoplasts: Examination of thin sections of lysozyme-induced spheres of B. subtilis with the electron microscope indicates that these bodies are completely devoid of cell wall.** We therefore consider use of the word protoplast appropriate,[16,17] although additional chemical, physiological, and immunological tests to demonstrate absence of residual wall material remain to be made.

Priming: This word is used in the sense of pump-priming. It carries no implications concerning the size of the molecular species and/or physical structure responsible for the priming process (i.e., the primer).

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* Landman; unpublished results.

** Ryter, A., and Landman, O.E; to be published, 1963.

III. MATERIALS AND METHODS

A. STRAINS

Strain 168 (indole$^-$) of Bacillus subtilis[18] was used throughout.

B. MEDIA

Bacterial inocula were grown on Difco Blood Agar Base medium. Stock cultures were maintained on potato extract agar slants[18] enriched with 20 grams of enzymatic casein hydrolyzate per liter and two grams of yeast extract per liter. To prepare cells for lysozyme treatment, two liquid media, SL1 and SL2, were employed.

SL1*	Grams/Liter
K_2HPO_4	14
KH_2PO_4	6
$(NH_4)_2SO_4$	2
Sodium citrate $.2H_2O$	1
$MgSO_4.7H_2O$	0.2
Glucose**	5.0
L-Tryptophan	0.05
Acid-hydrolyzed casein	0.2

SL2* — SL2 is identical to SL1 in its first six ingredients. In addition, it contains the following substances:

L-Tryptophan	0.005 grams per liter
Acid-hydrolyzed casein	0.1 gram per liter
$MgSO_4$	0.01 M
EDTA pH 7.0	0.0001 M
Sucrose	0.5 M

* SL1 and SL2 are based on the media used by Young and Spizizen[19] for transformation experiments with B. subtilis 168.
** Autoclaved separately.

The following fluid (DF) was used to dilute protoplast suspensions:

DF	
Sucrose	0.25 M
Sodium succinate pH 7.0*	0.25 M
EDTA pH 7.0	0.001 M
K_2HPO_4	0.02 M
KH_2PO_4	0.011 M
$MgCl_2$	0.01 M

* Sodium succinate is prepared as a 2 M stock solution by neutralizing succinic acid (Eastman) with equimolar quantities of sodium hydroxide.

A complex medium (C) and a chemically more nearly defined medium (SD) were used for plating of both protoplasts and bacteria.

C Medium

Brucella Broth*	28 grams per liter
Agar	9 grams per liter**
Defibrinated horse serum	100 ml per liter**
Sodium succinate pH 7.0	0.5 M
$MgCl_2$	0.005 M
$FeSO_4$	0.005 M
$CaCl_2$	0.001 M
$MnCl_2$	0.001 M

* Albimi Co., Brooklyn, N.Y.
** More recently, a modified C medium containing seven grams of agar per liter and five milliliters of serum per liter has been used.

SD Medium

Glucose	2 grams per liter
L-Tryptophan	0.02 gram per liter
NH_4NO_3	1 gram per liter
K_2HPO_4	3.5 grams per liter
KH_2PO_4	1.5 grams per liter
Agar	9 grams per liter
Defibrinated horse serum	5 ml per liter
Sodium succinate pH 7.0	0.5 M
$MgCl_2$	0.005 M

Generally speaking, these media are prepared by adding sterile concentrated stock solutions of the various ingredients to agar solutions of appropriate concentration at 48°C. In experiments where it was desired to assess the effect of osmotic stabilization, platings on C medium were compared with platings on an identical medium containing 1/10 the sodium succinate concentration (0.1 C).

C. METHODS

To prepare suspensions of L-colonies, small agar blocks containing counted numbers of colonies were excised from plates with a small spatula (made by hammering flat a piece of platinum wire) and placed into a few milliliters of suspension fluid (SF).

SF

Defibrinated horse serum	100 milliliters per liter
$MgCl_2$	0.01 M
Sodium succinate pH 7.0	0.5 M

The agar blocks in SF fluid were then drawn up vigorously into a syringe through a 20-gauge needle five inches long. Once all visible agar blocks had been disintegrated, the suspension was passed through the needle five more times to complete the breakup of L-form clusters.

Plates inoculated with protoplasts or L-colony suspensions were incubated at 26° or 30°C. Initially, incubation was carried out in evacuated desiccators, until we found that this procedure offered only marginal improvement over aerobic incubation.

IV. RESULTS

A. CONVERSION TO THE L-FORM

In our study of the conversion of bacilli to L-colony-forming elements, we have pursued the dual objectives of preserving viability and obtaining conversion rapidly. We have found that L-colony-forming elements may be obtained from both stationary and log-phase cells grown in either minimal or complex media. Further, a wide variety of media may be used in such conversion experiments. Both 0.5 M sucrose and 0.5 M sodium succinate, pH 7.0, are suitable osmotic stabilizing agents, but conversion is markedly slower in succinate and, for a given conversion rate, much more lysozyme is required in succinate than in sucrose media. After much empirical experimentation, the following procedure has been adopted because it has consistently given high yields of L-colony-forming elements and because it can be used simultaneously for transformation experiments.[20] Bacteria grown overnight on Difco Blood Agar Base plates are suspended in SL1 medium to a density of approximately 1.5×10^8 bacteria per milliliter and grown for four hours at 37°C with aeration to a density of approximately 5×10^8 bacteria per milliliter. This culture is diluted 1:9 into the sucrose-stabilized SL2 medium and incubation is continued for 90 to 120 minutes more. Now lysozyme is added and dilutions of the suspension are plated at intervals, using DF fluid as diluent. A set of curves from such an experiment, based on duplicate platings on C medium, are shown in Figure 1. It is seen that, at 200 micrograms of lysozyme per milliliter, more than 90 per cent of the bacillary colony-forming elements are lost during the first 20 minutes, while a corresponding number of L-colony-forming elements make their appearance. At 50 micrograms of lysozyme per milliliter, commitment of 90 per cent of the cells to the L-state requires about 40 minutes. At both enzyme levels, the entire original cell population was apparently converted to the L-state. The slight rise in count is peculiar to the particular experiment shown. Ordinarily, neither the bacterial control count nor the L-count exhibit such an increase, presumably because growth is inhibited in SL2 medium by tryptophan limitation, hypertonicity, and EDTA. Once lysozyme is present, division is prevented. However, a slight rise in count in the presence of lysozyme could be due to the formation of two protoplasts from some two-cell rods in the parent bacillary population.[7]

The residual bacillary counts at 50 and 200 micrograms of lysozyme per milliliter, shown in Figure 1, are obtained by counting the bacillary colonies that appear on C medium alongside the L-colonies. If the same suspensions are plated on a medium of lower osmolality, such as 0.1 C medium, this residual count is greatly reduced (Table I). Evidently, lysozyme attack renders the cells osmotically sensitive even before they are committed to the L-state. Concurrent microscopic observations indicate that cells retain their rod shape during this osmotically sensitive phase.

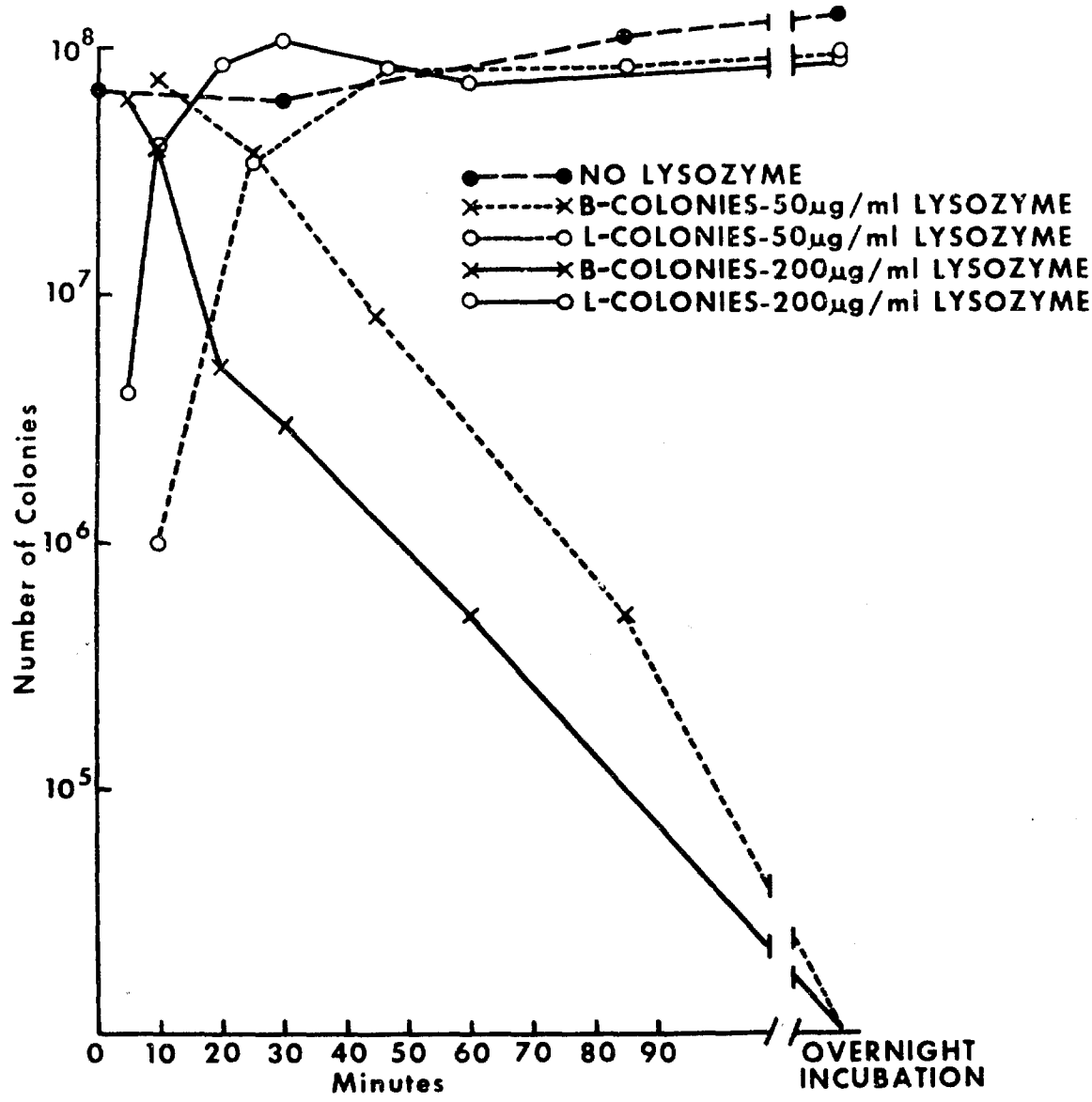

Figure 1. Commitment of B. subtilis Bacilli to the L-State in the Course of Lysozyme Treatment.

TABLE I. OSMOTICALLY SENSITIVE BACILLARY COLONY-FORMING ELEMENTS ARISING IN THE COURSE OF LYSOZYME TREATMENT OF B. SUBTILIS

Time After Lysozyme Addition, minutes	Lysozyme, μg per ml	BACILLARY SURVIVORS			
				Osmotically Sensitive	
		Total,[a] 10^7 cells A	Osmotically Insensitive,[b] 10^7 cells B	Number, A-B C	Fraction of Original Population[c] C/7.0 D
0	0	6.8	7.0	nil	nil
10	50	7.3	3.7	3.6	0.52
25	50	3.7	0.6	3.1	0.44
45	50	0.8	0.2	0.6	0.09[d]
85	50	<0.05	<0.05	---	--- [d]
5	200	7.5	0.7	6.8	0.99
15	200	2.2	0.3	1.9	0.28[d]
25	200	0.2	<0.05	0.2	0.03[d]

a. Count on C medium.
b. Count on 0.1 C medium.
c. Based on 0 time value in Column B.
d. The bulk of these populations has been converted to the L-state.

We suppose that the osmotic death of the rods on low-tonicity media is due to local rupture of the cell membrane in parts of the cell where the retaining wall has been weakened by lysozyme action.

B. GROWTH AND PROPAGATION OF L-COLONIES

A condition critical for the demonstration of lysozyme-induced commitment to the L-state has been the development of media for growing L-forms of B. subtilis with high plating efficiency. A large number of experiments have been devoted to the improvement and simplification of the complex media on which B. subtilis L-colonies were first recovered. This work is now near completion with the development of a defined medium that permits efficient assay of the viable elements. The composition of this medium is the same as that of medium SD except that the defibrinated horse serum has been replaced by 0.1 per cent crystalline bovine albumin or 2 per cent gelatin (Table II). On this defined medium, and similar media such as SD, an inoculum of either protoplasts or suspended L-bodies will give rise to L-colonies visible under 15X magnification in the dissecting microscope in three to four days at 30°C. On the complex medium C, colonies can be counted after approximately 30 hours. These rapid growth rates suggest that many elements in each colony are multiplying and that, in contrast to other L-forms, B. subtilis L-colonies contain many viable elements. Figure 2 relates viability data to colony size for bacillary colonies and L-colonies grown on modified C medium. All colonies were suspended by the syringe method and viable counts were made on modified C medium. It will be noted that the overlap between the B-colony and L-colony curves is not very extensive. This is due, on the one hand, to the difficulty of assigning accurate diameters to very small B colonies on C medium because of their rough morphology and, on the other hand, to the limit set by natural growth to the size of L-colonies obtainable on C medium (Figure 3). Despite these limitations, Figure 2 indicates that the relationship between colony diameter and viable number is quite similar in B- and L-colonies. It is concluded that much of the protoplasm of the B. subtilis L-colonies is contained in viable elements.

When the viable count data shown in Figure 2 are plotted semilogarithmically against time rather than colony size, a marked contrast between the growth curves of B- and L-forms emerges. The B-form curve is exponential until a colony size of 10^6 bacteria per colony is reached, while the growth rate of the L-forms declines sharply beyond 10^3 viable units per colony (Figure 3). These contrasts may be a reflection of the presumed difference in mode of replication between B-forms and L-forms.

Once an L-colony has been formed it can be propagated in the L-state indefinitely, if transfers are made before the onset of reversion. The onset of reversion, in turn, depends mainly on the media used. At present, an L-culture isolated eleven months ago is being passed through its sixty-fifth transfer.

TABLE II. GROWTH OF L-COLONIES ON DEFINED MEDIA CONTAINING DIFFERENT PROTEIN SUPPLEMENTS

Protein Supplement	L-COLONY SUSPENSION[a] INOCULUM		PROTOPLAST INOCULUM[b]	
	Number of Colonies[d]	L-Colony Diameter, mm[c]	Number of Colonies[d]	L-Colony Diameter, mm[c]
0.5% Horse Serum (SD Medium)	394 384	0.2	554 495	0.25
2% Horse Serum	373 300	0.3	497 435	0.40
0.5% Dialized Horse Serum	350 355	0.2	452 430	0.25
2% Gelatin	346 359	—[e]	535 482	0.33
2% Washed Gelatin	244 200	0.05	222 194	0.13
0.05% Gelatin	51 46	—	71 20	0.09
0.1% Crystalline Albumen	330 332	0.05	489 506	0.13
0.025% Crystalline Albumen	278 288	0.05	494 390	0.13
0.005% Crystalline Albumen	240 239	—	260 238	0.13
No Supplement	11 16	—	3 6	
C Medium	338 327	0.4	385 378	0.60
SD Medium + 0.2% DL-asparagine	290 270	—	435 377	0.28

a. The L-colonies were suspended in DF fluid to avoid contamination with serum.
b. Protoplasts from a 30-minute lysozyme treatment were used. Prior to lysozyme addition, the bacterial count was 700.
c. After 4 days at 30°C.
d. Total number of colonies; i.e., bacillary colonies (B) + L-colonies (L). In both experiments, platings on DL-asparagine medium and early observations showed that the entire inoculum was in the L-state. B colonies observed at the time of counting were therefore due to reversion.
e. Colony sizes on these plates were so varied that no representative average colony diameter can be given.

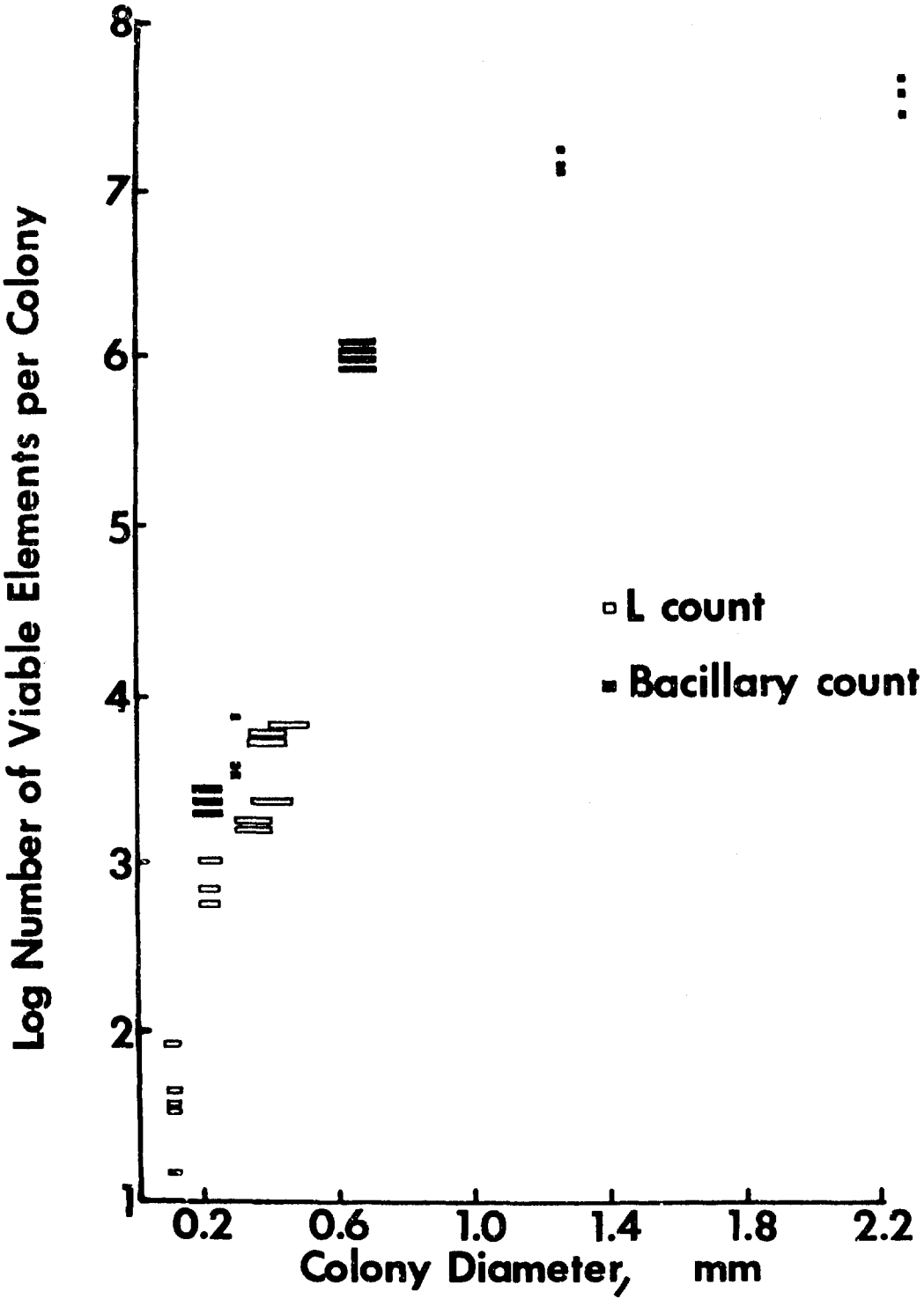

Figure 2. Number of Viable Elements in Bacillary Colonies and L-Colonies of Different Sizes on Modified C Medium.

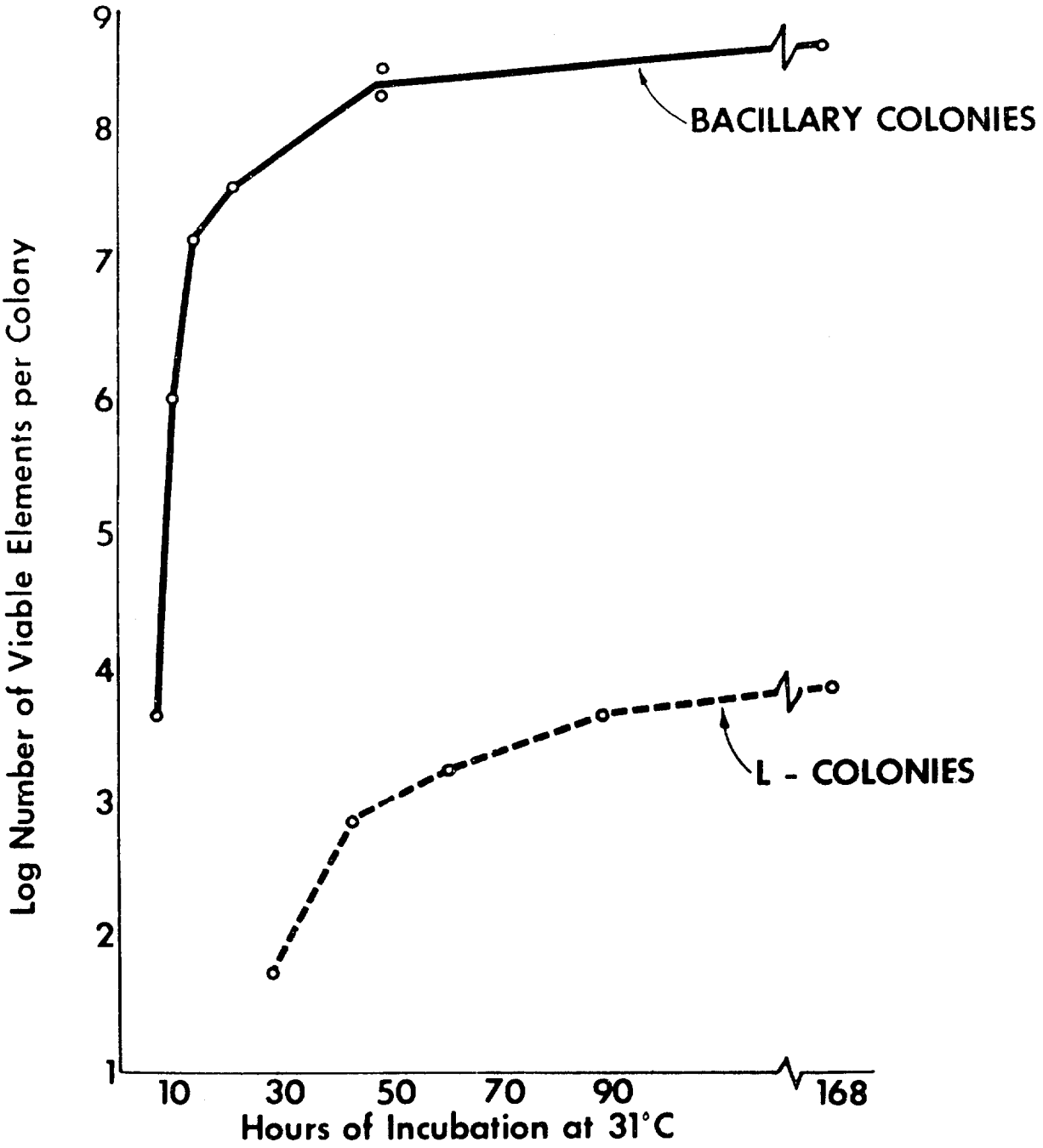

Figure 3. Growth Rate of Bacillary Colonies and L-Colonies on Modified C Medium.

C. REVERSION

In contrast to the stable mass-conversion L-forms of Salmonella, E. coli, and Streptococcus, B. subtilis L-forms fevert quite readily to the bacillary form. This makes it possible to study the factors that enhance or retard reversion. In most experiments so far, the procedure has been to look for either accelerated or delayed overgrowth of L-colonies on SD medium by bacillary revertants in response to additives or altered growth conditions. Results of an experiment with reversion inhibitors are presented in Table III. In the experiment shown, an L-colony suspension in SF fluid was spread onto plastic Petri dishes containing SD agar with additives. Three days after the start of incubation at 30°C, and again after 4, 6, 10, 12, and 14 days, the colonies were counted and classified as either L or reverted L on the basis of their morphology and of phase-contrast microscope examinations of squashes of typical colonies. The data of Table III demonstrate that D-serine, D-threonine, D-phenylalanine, D-methionine, D-asparagine, and DL-pantoyl lactone + D-asparagine all delay the onset of reversion. Pantoyl lactone enhances inhibition by D-asparagine. As shown for D-phenylalanine, the higher concentrations of the D-amino acid delay reversion more effectively than lower concentrations. They also tend to inhibit growth more, but growth inhibition in general cannot account for reversion delay. This is obvious from a comparison of the colony diameter data with the reversion delay data of Table III. Further, there are some D-amino acids, for instance D-tryptophan, that inhibit growth markedly but do not inhibit reversion at all. Since amino acids have been reported to induce the formation of L-forms[21,22] and spheroplasts,[23] it becomes pertinent to inquire whether the D-amino acids, at the concentrations employed, are merely maintaining the L-state in B. subtilis or whether they are actually inducing it. This question is answered by the observation that only B colonies are formed when bacilli are inoculated on the D-amino acid media (Table III).

Reversion inhibition by D-amino acids is scarcely affected by the presence of their L-isomers. A comparison of the data for D-methionine and DL-methionine illustrates this point (Table III). On the other hand, growth inhibition by D-amino acids is ameliorated by the L-isomers. For example, both growth and L-colony stability are very good in SD medium containing two grams per liter DL-asparagine, while growth is almost nil in the same medium base with one gram of D-asparagine per liter. The reversion-inhibiting properties of D-amino acids were first noted in an experiment in which DL-asparagine was used as a nitrogen source. DL-asparagine has been used extensively when preservation of committed forms in the L-state is desired.

Among the various factors tested for ability to increase the reversion rate, chemical additives have so far given only inconsistent results. More decisive, and of great interest, is the effect of the physical properties of the medium on reversion. Specifically, it has been found that the agar concentration strongly influences the reversion rate. Thus, when C or SD

TABLE III. L-COLONY REVERSION PATTERN ON SD MEDIA CONTAINING ADDITIVES

Additives, grams/liter	BACILLARY INOCULUM		L-SUSPENSION INOCULUM							
	Count, 5th day	B-Colony Diameter On 5th Day, mm	Total (B + L) Count, 4th day	L-Colony Diameter On 6th Day, mm	Per Cent L-Colonies Showing Bacillary Overgrowth After Incubation At 30°C for No. Days Shown					
					3	4	6	10	12	14
None	127		453		8.6	15.4	29.4	99.6		
	113	1.25	494	0.45		16.4	31.6	94.6		
	129		499			27.8	33.8	91.3	100	
D-serine, 0.1	122		434		1.6	2.1	9.2	34.5	62.7	64.4
	122	0.80	544	0.40		3.5	7.7	30.0	68.4	68.4
	119		489			4.7	11.9	47.3	71.7	76.6
D-threonine, 0.1	122		494		0	2.4	7.3	13.2		34.5
	111	0.30	545	0.25		0.2	0.6	0.9	3.5	
	113		547			0	0	0.6		2.6
D-phenylalanine, 0.1	127		453		0.2	0.4	0.7	21.4	59.6	62.0
	129	1.80	529	0.55		0.2	1.4	22.8	56.5	68.0
	123		462			0.7	1.1	16.2	47.5	60.6
D-phenylalanine, 0.3	113		517		0	0	0.6	4.3		11.6
	116	1.70	535	0.60		0	0.2	3.0	6.2	11.0
	138		575			0.2	0.2	3.0		4.9
D-methionine, 0.6	107		596		0	0	0	0.8	1.7	1.7
	119	1.65	557	0.45		0	0	0.5	1.8	1.8
	116		492			0	0.2	1.0	1.4	1.4
DL-methionine, 1.2	119		296		0	0	0			
	115	1.15	542	0.30		0	0	3.1	7.4	
	106		587			0	0	1.2	1.2	1.4
D-asparagine, 0.4	113		320		0	0.6	28.8	95.3	100	
	106	0.60	291	0.30		2.8	14.8	86.6	90.7	93.8
	135		462			1.3	16.0	91.1	98.5	100
DL-pantoyl lactone, 2.5	114		511		8.0	34.1	47.5	100		
	115	1.15	621	0.35		20.3	22.0	98.6	99.2	
	115		433			19.8	31.6	81.0	86.9	93.8
DL-pantoyl lactone, 2.5 + D-asparagine, 0.4	127		486		0	0.2	15.4	67.0	74.0	82.1
	119	.70	463	0.35		0.2	7.3	70.5	77.5	79.0
	118		483			0.2	1.4	40.0	43.0	57.7
D-tryptophane, 0.3	102		438		2.3	12.8	21.5	100		
	110	1.50	361	0.30		22.8	30.9	100		
	104		474			15.4	21.3	100		

media containing 20 or 25 grams of agar per liter are inoculated with protoplast suspensions or with L-body inocula, L-colony growth does not occur. Instead, bacillary colonies soon begin to appear at the site of inoculation. Further, if the agar concentration is lowered to seven grams per liter, the rate of reversion falls well below that observed with the customary concentration of nine grams per liter. A photographic record demonstrating the role of agar in reversion is shown in Figure 4. In the experiment shown, single L-colonies growing on SD medium containing D-asparagine and seven grams of agar per liter (our standard medium for maintaining L-forms in successive transfers) were cut out of the agar in small blocks and inverted on C medium plates containing 7, 9, and 25 grams per liter of agar. The blocks were then dragged face down over the agar toward the center of the plates. Each agar block left behind a trace of inoculation. After 48 hours of incubation at 30°C in evacuated vacuum desiccators, the pictures shown in Figures 4a through 4e were taken. At the same time, B- and L-colony counts of the "drags" were made; these are given in Table IV. Figure 4 and Table IV show that viable elements that give rise to L-colonies on soft agar give rise to bacillary colonies on hard agar.

Until microscopic studies can be made, we are unable to state whether any multiplication of L-forms occurs in hard agar prior to the reversion event, or whether reversion is induced in the inoculated L-body directly. The rapid rate of appearance of the revertant colonies suggests, however, that reversion occurs quite soon after plating. Whatever the precise timing of reversion, we have not succeeded in efforts to obtain it in all of the viable bodies inoculated on hard agar. When an inoculum of known count is used (e.g., a suspension of recently formed protoplasts) usually less than 15 per cent of the inoculated bodies give rise to colonies on hard agar.

Conditions suitable for reversion in hard agar are not necessarily optimal for growth of L-forms. Thus, when plates are incubated at 35°C, the survival of L-bodies on soft agar is erratic and often low; their reversion on hard agar is usually not sensitive to the temperature change. Consequently, bacillary colony counts on 2.5 per cent agar are often higher than L-colony counts on 0.7 per cent agar when plates inoculated with a given L-colony suspension are incubated at 35°C.

Although the remarkable effect of hard agar on reversion deserves further study, recent experiments have focused on reversion in yet another system, gelatin media: When an L-colony suspension or protoplast suspension is plated on SD medium containing 15 to 35 per cent gelatin instead of 0.9 per cent agar, reversion is both rapid and efficient. Usually, the number of bacillary colonies counted on gelatin media after 36 hours is equal to the number of L-colonies developing on SD medium. Nearly 100 per cent of the inoculated protoplasts or L-bodies are thus capable of reversion in the gelatin medium (Table V). As in the case of hard agar, L-colonies of visible size do not develop on gelatin media— only bacillary growth is observed with the dissecting scope.

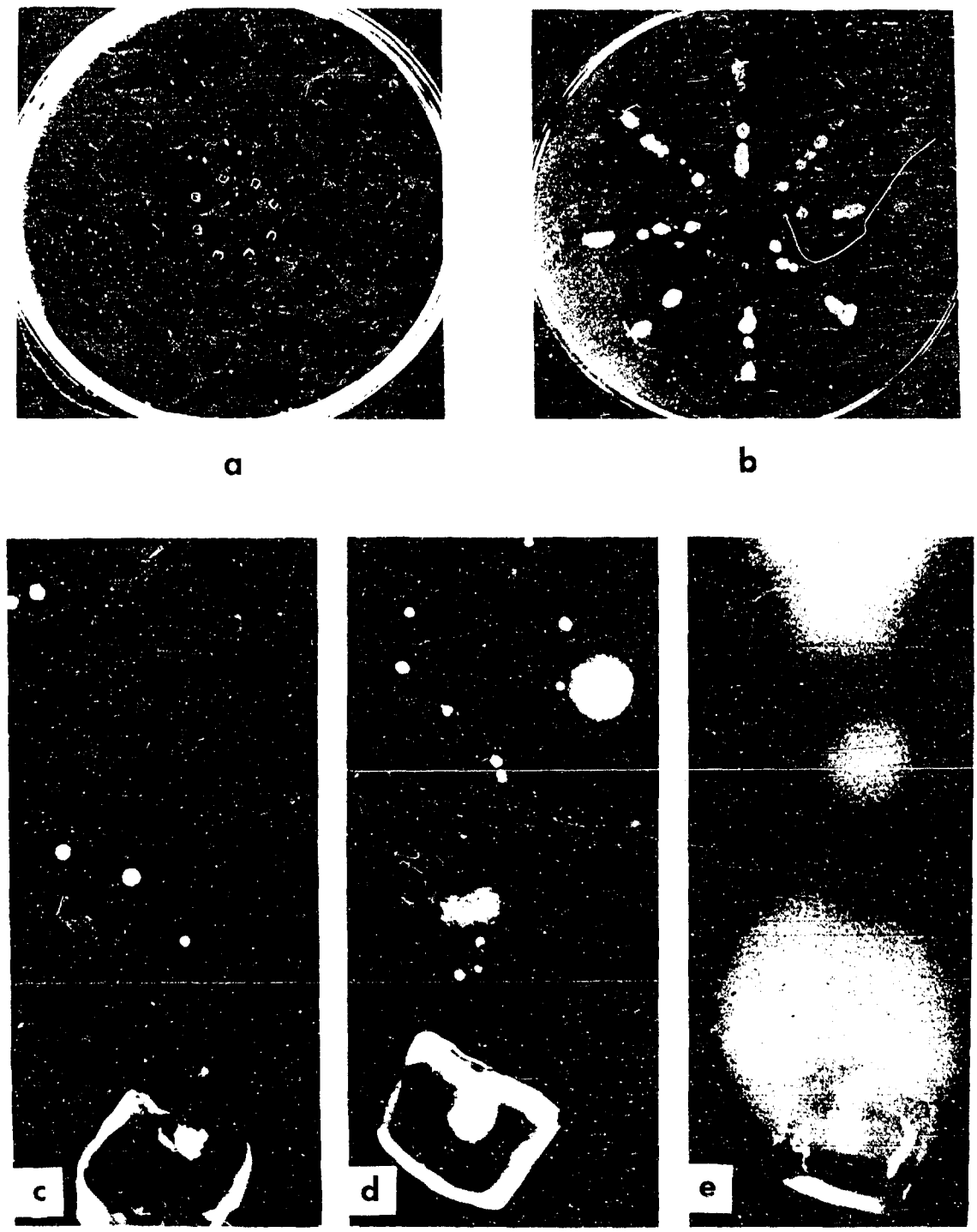

Figure 4. Effect of Agar Concentration on Reversion. Agar blocks containing single L-colonies were inverted in a circle on plates containing C medium with 0.7 (a), 0.9 (not shown), and 2.5 (b) per cent agar. Blocks were dragged across the agar toward center of plate, leaving a line of inoculation. After 48 hours' incubation at 30°C, photos of entire plates (a and b) and closeups of representative streaks on 0.7 (c), 0.9 (d), and 2.5 (e) per cent agar were taken. Several L-colonies on (d) had recently reverted.

TABLE IV. REVERSION ON C MEDIA CONTAINING DIFFERENT AGAR CONCENTRATIONS[a]

Drags on 0.7% Agar		Drags on 0.9% Agar		Drags on 2.5% Agar	
L	B	L	B	L	B
13	0	29	8	0	6
33	0	28	11	0	7
37	0	23	12	0	7
39	0	31	17	0	6
37	0	17	9	0	5
26	0	8	4	0	9
40	0	17	5	0	7
24	0	35	16	0	3

a. Counts shown are the numbers of colonies in individual "drags" (see Figure 4) and their classification as either L, or reverted L (B), 48 hours after inoculation.

The efficiency and rapidity of reversion in gelatin media makes it possible to study reversion in detail. Work with this system is just beginning, but it is already evident that reversion may occur as early as five hours after contact with medium containing 30 per cent gelatin. As shown in Table V, recently formed protoplasts or L-bodies derived from a parent cell committed nine months previously revert with equal efficiency in gelatin.

D. PENICILLIN L-FORMS OF B. SUBTILIS

According to our present views on L-forms, the crucial event in the transition from bacillary form to stable L-form is the loss of a priming system for both septation and cell-wall formation. This loss may be induced by a variety of treatments:[24] by enzyme treatment as described above, by streptomycin starvation in streptomycin-dependent strains,[6] by nutritional deprivation,[25] or, most commonly, by penicillin treatment.[3,24]

TABLE V. REVERSION FROM L-STATE AND FROM PROTOPLAST STATE ON DEFINED MEDIA

MEDIUM[a]	L-SUSPENSION INOCULUM[b]		30-MIN PROTOPLAST[b] INOCULUM	
	B-Colonies[c]	L-Colonies	B-Colonies[c]	L-Colonies
15% Gelatin	443, 431	0, 0	211, 346	0, 0
20% Gelatin	369, ---	0, ---	287, 269	0, 0
25% Gelatin	486, 429	0, 0	406, 375	0, 0
30% Gelatin	382, 470	0, 0	294, 324	0, 0
35% Gelatin	453, 362	0, 0	506, 498	0, 0
Modified C Medium	117, 150	291, 250	130, ---	200, ---
SD Medium + 0.2% DL-Asparagine	0, 0	370, 410	0, 0	376, 435
0.1 C Medium	---, ---	---, ---	0, 0	0, 0

a. All gelatin media are made up in SD base lacking agar and serum. Serum is omitted from these media because even two per cent gelatin satisfies the requirement for protoplast stabilization (Table II). The phosphates are used at one-fifth their normal concentration to avoid formation of a precipitate. The gelatin used here was not washed, but gelatin washed by three successive ethanol precipitations also gave efficient reversion.

b. The L-forms used for this experiment had been transferred in the absence of inducing agent (lysozyme) for nine months (57 transfers). The protoplasts had all been in the B state 30 minutes prior to plating. The count of the bacillary suspension before protoplasting was 700.

c. Counts shown were made after six days of incubation at room temperature. Counts made three days after inoculation gave values ranging from 59 to 91 per cent of the counts shown. At that time, all colonies on gelatin were bacillary, all colonies on DL-asparagine were L, and about 96 per cent of the colonies on modified C Medium were L.

If this view is correct, the nature of the inducing agent should not affect the properties of the resultant L-forms once they are compared in identical environments. To test this hypothesis, the properties of penicillin-induced L-forms of B. subtilis were studied. Bacilli were plated on C media containing 0, 0.1, 1, 10, 100, and 1000 units of penicillin per milliliter, and the survival (in the L-state) at each penicillin concentration was determined (Table VI, column A). L-colonies from each medium were then excised and transferred several times on SD medium containing 0.2 per cent DL-asparagine. A lysozyme-induced L-culture was transferred alongside. The reversion behavior of the lysozyme-induced L-forms and L-forms induced by 0.1 unit penicillin per milliliter and 1000 units penicillin per milliliter was then compared on media that permitted, respectively, rapid, slow, and delayed reversion. The results indicated that the reversion pattern of the three different L-form types was very similar (Table VII). Further examination of colony morphologies did not disclose any differences between L-forms of differing origin. On the basis of the morphological observations, and of the reversion results, we have concluded that the penicillin-induced L-forms of B. subtilis are probably the same as the lysozyme-induced L-forms.

If, instead of bacteria, penicillin-induced L-forms are plated on penicillin-containing C media, the resistance pattern in column B of Table VI is obtained. Comparison of columns A and B of Table VI reveals that the pattern of penicillin resistance of the L-bodies differs from that of the bacteria (or rather, from that of the bacteria turning into L-forms). With more than one unit of penicillin per milliliter, the L-forms are evidently more penicillin-resistant than the converting B-forms. With this finding, it may be said that the B. subtilis inheritance system described here possesses the principal hallmarks of Lamarckian inheritance: An unfavorable environment, penicillin, induces a heritable change in the individuals subjected to it; the individuals that have acquired the new heritable characteristics are then more resistant to the unfavorable environment than their parents.

TABLE VI. PLATING EFFICIENCY OF BACTERIA AND L-SUSPENSIONS ON MEDIA CONTAINING GRADED CONCENTRATIONS OF PENICILLIN

PENICILLIN CONCENTRATIONS, units/ml	PER CENT SURVIVAL OF INOCULUM			
	A		B	
	Bacterial Inoculum		L-Form Inoculum	
	Range[c]	Average[a]	Range[c]	Average[b]
0		100		100
0.1	16 - 54	31.5	5 -25	17.0
1.0	0.1 -7.6	3.4	6 -31	15.2
10.0	0.2 -1.9	0.86	5 -32	15.6
100.0	0.03-1.2	0.58	6 -35	14.0
1000.0	0.15-0.93	0.37	0.6-31	13.0

a. Seven experiments.
b. Five experiments.
c. Although the absolute recoveries varied sharply from experiment to experiment, each individual B-inoculum experiment showed a sharp drop-off of recoveries as the penicillin concentration was raised, while each individual L-inoculum experiment gave fairly uniform recovery at all drug levels.

TABLE VII. COMPARISON OF THE REVERSION RATES OF PENICILLIN-INDUCED AND LYSOZYME-INDUCED L-FORMS ON THREE DIFFERENT MEDIA

L-Form Culture[a] Obtained Through Induction By	Per Cent Revertants After Incubation at 30°C For No. of Days Shown							Reversion Test Plating Medium
	2	3	5	6	7	8	9	
Lysozyme	3.0	4.8	56.3	55.8	89.0			C Medium Base +
0.1 unit per milliliter penicillin	0.9	13.6	94.5	97.8	99.5			0.9% Agar +
1000 units per milliliter penicillin	4.9	22.6	97.8	100				0.5% Serum
Lysozyme	5.3	5.4	10.4	9.8	18.0	---	43.0	C Medium Base +
0.1 unit per milliliter penicillin	4.8	5.5	9.2	10.1	13.0	---	30.0	0.7% Agar +
1000 units per milliliter penicillin	3.2	6.9	8.9	11.4	15.5	---	53.7	0.5% Serum
Lysozyme	---	0	0	1.9	2.2	43.6	44.8	SD Medium +
0.1 unit per milliliter penicillin	---	0	0	2.5	3.1	60.3	66.3	0.2% DL-
1000 units per milliliter penicillin	---	0	0.5	2.6	7.2	71.3	65.1	Asparagine

a. All three L-form types were derived from the same single-colony isolate of B. subtilis 168 and all were transferred on SD medium + 0.2% DL-asparagine prior to use in this experiment. The lysozyme-induced L-culture was transferred six times, the culture originally induced by the 0.1 unit of penicillin per milliliter was transferred three times, and the culture originally induced by the 1000 units of penicillin per milliliter was transferred once.

V. DISCUSSION

The initial objective of this study was to demonstrate (a) that lysozyme protoplasts of Gram-positive bacteria, like the stable L-forms of Gram-negative bacteria, lack a septation-and-wall-formation primer, and (b) that the normal septation process can be bypassed if the appropriate soft agar is provided. Quantitative rescue of B. subtilis protoplasts from death and their survival as L-colonies in soft agar has provided satisfying support for these postulates. Shortly after our experiments were initiated, Gooder and Maxted[26] published parallel results, which showed that up to 10 per cent of enzymatically produced protoplasts of streptococci formed L-colonies on agar media. Earlier, Eddy and Williamson[27] had found that yeast protoplasts produced by the action of a snail-stomach enzyme were capable of reproduction in the wall-less state on agar media. Bachmann and Bonner[28] showed that protoplasts of Neurospora, produced by the same snail enzyme, often grew into clusters of spheres before reverting to the walled state.

Although these various studies were all undertaken with different assumptions and objectives, each one demonstrates a partial and sometimes persistent heritable inability of microbial protoplasts to reinitiate the division-and-wall-forming functions of the cell, once the cell wall has been removed enzymatically. The most obvious interpretation of these facts is that removal of the wall deprives the cell of a component of a self-sustaining (feedback) reaction chain that is required for septation-and-wall-formation. From the fact that commitment occurs when the cell wall is removed, we infer that this component, the primer, is located at the periphery of the cell, perhaps in the cell wall, but more probably at the cell membrane.[3,26] The data presently available do not permit a final judgement concerning the nature of the primer. A. priori, an enzyme, a metabolic intermediate, a cell-wall fragment, an RNA messenger, and a variety of other components could all serve as indispensable links in a feedback chain and hence as primer.

Whatever the nature of the priming activity, its heritable loss due to cell-wall removal seems to coincide with the appearance of protoplast morphology. A state of osmotic instability, which is also a consequence of incipient cell-wall removal, precedes commitment to the L-state and the appearance of protoplasts.[29]

Once suitable methods for their recovery and propagation had been developed, the B. subtilis L-forms were found to possess properties highly favorable to further study: Compared with the stable L-forms of Salmonella and E. coli, those of B. subtilis grow very rapidly and exhibit excellent viability. As a result, it has been possible to develop defined media for their propagation. Most important, the high spontaneous rate of reversion of B. subtilis L-forms offered renewed hope that the nature of the priming process might become accessible to experimental analysis.

Although the high spontaneous reversion rate and the very high induced reversion rate of B. subtilis L-forms operationally resemble the mass reversion of reverting L-forms (unstable L-forms, 3B forms; see Dienes and Weinberger[24]), a clear conceptual distinction between these two L-form types must be made. The reverting L-forms found in such Gram-negative bacteria as Salmonella, E. coli, and Proteus are phenotypes that are induced to L-morphology by the inhibitory action of drugs or reagents. When the inhibitory agent is withdrawn, the cells return to normal bacillary morphology. By contrast, stable L-forms are operationally defined in our laboratory as those forms that continue to give rise to L-colonies even when the inducing agent has been removed. Stable L-forms may arise as a result of gene mutation[5,14] but, more commonly, they are induced by a mass conversion process such as that described in this paper. The stable mass-conversion L-forms of different bacteria and bacterial substrains differ greatly in their tendency to reinitiate the bacillary state. Thus, under the conditions explored by the various investigators, the L-forms of E. coli "never" revert,* those of streptococci[26] and Salmonella[3] revert very rarely, those of staphylococci,[25] Proteus mirabilis,[15] and B. subtilis revert freely. As we have shown here, this tendency to revert is profoundly affected by both the chemical and physical environment.

Among observations with chemicals, the inhibition of reversion by D-amino acids relates interestingly to other recent publications on the effect of D-amino acids on bacterial morphology. In particular, Lark and Lark[23] have described the induction of spheres ("crescent forms") in Alcaligenes fecalis by D-methionine and 14 other D-amino acids. Although the Alcaligenes fecalis spheres have also lost their ability to divide, Lark and Lark focus their attention mainly on the inhibition of the formation of the rigid cell wall layer. Through a study of the incorporation of labeled D- and L-methionine into cell wall and protoplasmic fractions, they reach the conclusion that D-methionine blocks cell wall formation by interfering with the integrity of a cell wall primer (which they believe to be part of the rigid layer).[30] As in our system, L-methionine does not block D-methionine effects on cell-wall formation. The thorough investigations by Grula[31] with a species of Erwinia are concerned mainly with septation inhibition by D-amino acids, although concomitant spheroplasting effects are duly noted by that author. Grula and Grula[32] find that filament formation caused by six different D-amino acids can be reversed by pantoyl lactone, to a lesser extent by metal ions such as Zn^{++}, Ca^{++}, and Mn^{++}, and by other agents. The L-isomers of the D-amino acids generally do not counteract the effect of their D-isomers. In sum, there is clear prior evidence for inhibition of both cell-wall formation and septation by D-amino acids. Our observation, that D-amino acids prevent reversion of stable L-forms, indicates that this inhibition is exerted at a site that

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* Landman; unpublished results.

is involved in the priming of both of these cellular functions. Beardsley,[21] who has observed the induction of L-forms by D-amino acids, has come to the same conclusion. Scattered data of our own, and the results of Grula and Grula[32] and of others in this field[33] suggest that the metal ions also strongly influence the functioning of the priming mechanism under consideration.

In contrast to the rather extensive literature on inhibition of septation and cell-wall formation by chemical reagents, the effect of the physical characteristics of the medium on these cellular functions has scarcely been investigated. The sole exception of which we are aware is a report by Necas that yeast protoplasts revert efficiently when they are imbedded in gelatin media.[34,35] His observations, and our own quantitative data on agar- and gelatin-induced reversion, carry suggestive implications concerning the mechanism of reversion. For one, the vast chemical difference between agar and gelatin practically rules out any speculation that either of these impure materials could be furnishing the same priming substance to the L-bodies. Further, the observation that a threefold increase in agar concentration from 0.7 per cent to 2 per cent changes the reversion pattern of protoplasts from rare to very frequent also speaks against the idea that agar might be furnishing a priming substance. Finally, the findings that reversion is similar in Difco Noble Agar, Difco Purified Agar, and Difco Bacto-Agar, and that gelatin purified by three ethanol precipitations exhibits about the same reversion stimulation properties as unwashed gelatin, also suggest that it is the physical properties of the gels rather than their chemical constituents that promote reversion.[34,35]

If agar and gelatin act physically and do not furnish the L-bodies with a priming substance, the cells themselves must produce all necessary products and information for the reinitiation of the bacillary state. The fact that all of the L-bodies plated on gelatin medium can give rise to bacterial colonies further shows that this potential is inherent in each viable L-body. Somehow, this potential can be realized in each L-body in a few hours in gelatin media. On soft agar, on the other hand, reversion is observed only after a lengthy incubation period and only after each L-body has given rise to numerous progeny. One of these offspring then gives rise to the revertant, which overgrows the parent L-colony. It appears that the L-bodies are actually (or potentially) producing primer all the time but that the primer can be retained by the cells or become functional far more efficiently in hard agar or gelatin than in soft agar. The primer must therefore be of such a nature that the difference in medium consistency can spell a difference to its retention or formation by the cell. In particular, we may ask what kinds of molecules may be expected to be arrested in their diffusion by 2 per cent agar and 15 to 30 per cent gelatin, but not by 0.7 per cent agar; or, alternatively, what kinds of molecules might be expected to accumulate beyond a threshold priming concentration in the hard agar, but not in the soft agar? A survey of the relevant literature indicates that, barring a chemical or special physical interaction between the diffusing substance and the agar,[36] only elements in the 30 to 50-

millimicron size range are "arrested" in their diffusion as the agar concentration is raised to 2 per cent.[37,38] On the other hand, molecules generally are slowed in their diffusion rate in hard agar relative to their free diffusion rate. This effect is proportional to molecular size and not very large. For example, even in the case of bovine serum albumen, a protein of molecular weight 65,000, there is only a 28 per cent difference in rate between diffusion in liquid and in 1.5 per cent agar.[39] It therefore appears doubtful that the diffusion of a small molecule could be modified sufficiently by the hard agar to account for "threshold concentration" priming.

A widely held view concerning the nature of the primer postulates that the primer is a fragment of polymerized cell wall.[10,30] Earlier work in our laboratory with penicillin-induced L-forms of Salmonella paratyphi provides evidence against this idea: We have already mentioned that Salmonellae give rise to both reverting and stable L-forms. Reverting L-colonies are produced at 10 units of penicillin per milliliter but, on the same medium, only stable L-colonies emerge at 40 units of penicillin per milliliter. As judged by phage adsorption experiments, the reverting L-forms retain considerable amounts of wall material, apparently both lipopolysaccharide and lipoprotein layers,[40] while the stable L-forms appear to have lost all of this material.[2] Depolymerization of the rigid (mucopeptide) layer alone thus leads merely to a phenotypic alteration, the reverting L-form. By contrast, mass-conversion commitment to the stable L-state in Salmonella,[2] in Proteus,[41] in E. coli,[2] in Streptococcus,[26,42,43] and in B. subtilis seems to be correlated with the loss of the entire cell-wall structure. The fact that depolymerization of the rigid layer does not cause commitment in Gram-negative bacteria suggests that commitment in the Gram-positive B. subtilis may not be due to the loss of the rigid layer per se either. Instead, the apparent correlation between commitment and complete cell wall removal, regardless of the chemical constitution of the wall, focuses attention on the cell membrane or the space between wall and membrane as the site of localization of the primer.

Although the preceding considerations appear to direct the study of reversion toward the subject of macromolecule diffusion, the primer problem will not necessarily be solved by the pursuit of this approach. It is quite possible, for instance, that there is no diffusing macromolecule and that, instead, priming is due to a mechanical interaction between gelatin or hard agar and the L-bodies. One of the most attractive hypotheses of this type postulates that the element responsible for wall and septation priming is a mesosome—[44] that such membranous organelles are lost in the course of protoplast formation in B. subtilis, perhaps in the manner in which a wrinkle in an inner tube is lost when the tire is removed, and that new folding of the membrane is mechanically induced by those solid media that cause reversion.[45]

Several findings published in the literature lend support to the mesosome hypothesis; some others tend to contradict it. On the positive side, there is evidence that the mesosomes of Bacillus species are lost during protoplast formation.[44, -*] If the mesosomes were indeed identical with the primer, loss of mesosomes should always be correlated with commitment. Recent evidence that Micrococcus lysodeikticus protoplasts retain a good many mesosomes[46] therefore militates against the mesosome hypothesis, but cannot be considered conclusive, at least until it is shown that these protoplasts are committed to the L-state. The mesosomes are an attractive object for speculation in the present context because electron microscopy has implicated these organelles quite clearly in the septum-forming process[44,45,47,48] and, to a lesser extent, in wall formation.[49] An earlier version of the mesosome hypothesis has been published by Salton.[50] We have initiated collaborative electron microscope studies to test some of the predictions of this hypothesis.*

Viewing our studies on induced inheritance changes in the larger framework of genetics, the question arises as to whether the facile environmental modulation of inheritance that we have observed is confined to this rather pathological system in microorganisms or whether other inheritance systems display similar properties. The answer is, of course, that there are a variety of systems that bear a formal resemblance to the present one. The position that membrane inheritance in B. subtilis is destined to occupy among these systems depends largely on the outcome of studies on the nature of the priming mechanism. If the missing element in the feedback chain should turn out to be an escaping molecular species, our system could be classed as a dynamic equilibrium system.[12] (A dynamic equilibrium system in which inherited changes are triggered by transient changes in the medium has been described by Novick and Weiner.)[13] Alternatively, if the mesosome hypothesis were to be confirmed, the membrane inheritance of B. subtilis would be appropriately compared with more autonomous cytoplasmic inheritance systems such as the "cortical inheritance" system of Paramecium.[51]

Aside from considerations of mechanism, the system that bears the closest formal resemblance to ours is the inheritance of immobilization antigens in Paramecium.[52] The immobilization antigens are subject to mass conversion and mass reversion. These changes can be induced by a variety of environmental agents, including chemicals and enzymes. The antigens are located at the cell surface and their reponse to the environment is determined by the genes, just as the response of bacteria to the L-form-inducing action of penicillin is gene-controlled.[3,53] The inheritance of the immobilization antigens in Paramecium has repeatedly been cited as a model for the inheritance of cell-line differences in embryogenesis.[54,55] In both embryogenesis and the inheritance of immobilization;

* Ryter, A., and Landman, O.E.; to be published, 1963.

antigens, heritable changes persist in cells of identical genic composition; both systems respond to the chemistry and physics of the environment, and in both the cell surface appears to play a major role. The data cited here suggest that an inheritance system exhibiting parallel properties may already exist at the bacterial level.

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