CRITICAL FACTORS INFLUENCING GROWTH OF L FORMS OF *PROTEUS MIRABILIS*

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The observations, primarily by Dienes and associates, of L colonies arising spontaneously or by penicillin induction from species of *Proteus* or *Salmonella* led to the description of two morphologically distinct colonial forms, designated as the 3B type and the 3A type. It has been reported further that the viable elements of these two types exhibit markedly dissimilar properties such as size, nutritional requirements, and ability to return to the bacilliform state (Dienes and Weinberger, 1951). Biochemical and enzymological analyses of 3B L forms have supported the concept that these elements are essentially spheroplasts and possess the capabilities of the bacillary cells from which they are derived (Kandler, Zehender, and Müller, 1956). In contrast, however, 3A type L forms are deficient in many of the capabilities displayed by the parent cells. The manifold uncertainties concerning the genetic, morphological, and biochemical characteristics of L forms and pleuropneumonia-like organisms (PPLO) have been extensively detailed in a recent symposium (Liebermeister, 1960; Sharp, 1960; Medill-Brown, Hutchinson, and Cocklin, 1960).

Recent investigations (Landman, Altenbern, and Ginoza, 1958) have shown that a soft agar medium can be devised upon which every *Proteus mirabilis* cell plated would be converted to and would propagate as the 3B type of L form. There are no definitive data concerning conversion of bacillary cells to 3A type L forms. Preliminary qualitative experiments in this laboratory to determine conditions yielding the maximal number of 3A type L colonies from an inoculum of Proteus cells indicated that low penicillin concentration, low tonicity, and an incubation temperature of 30 C were favorable. Widely fluctuating quantitative results from seemingly identical experiments prompted a comprehensive examination of many factors possibly influencing growth of 3A type L colonies from an inoculum of Proteus cells. The data in the present paper establish the crucial importance of several unsuspected factors, which may partially account for the often encountered erratic nature of growth of L forms.

MATERIAL AND METHODS

The strains of *P. mirabilis* employed were recent isolates from clinical materials. They were maintained at 4 C on nutrient agar slants and transferred to fresh medium every 6 weeks. Unless otherwise specified, the strain F of *P. mirabilis* was routinely employed. Inocula of cells were prepared by overnight growth with aeration at 37 C in penassay broth (Difco). Such cultures containing about 5×10^9 cells per ml were decimally diluted in fresh penassay broth and plated by spreading 0.1 ml of appropriate dilutions on the surface of previously poured plates. The general medium notation, defining agar concentration, penicillin concentration, and molarity of sodium succinate, is the same as that already described (Altenbern and Landman, 1960). Unless otherwise specified, plates for the growth and enumeration of L colonies were incubated at 30 C for 6 days. The total viable cell count was determined by plating on medium I (agar, 0.8%; penicillin, none; succinate, 0.5%) followed by incubation at 30 C for 48 hr. L colonies were counted under a binocular dissecting microscope. The L colony counts of either morphological type refer to the number of L colony forming centers per ml of the original inoculum culture described above and are averages of triplicate platings.

A basal medium of the constituents of penassay broth was employed for all platings. When necessary, the penassay medium was prepared from concentrated solutions of the individual components of the medium. This procedure varied moderately in accordance with the design of individual experiments. To a 250-ml flask were added 10 ml of each of the following: peptone, 10% (w/v); yeast extract, 3%; beef

TABLE 1

Transformation of 3A type L colonies into 3B type L colonies during incubation on medium II

Incubation Temperature	Per Cent 3B Colony Types after Incubation for					Total L Colony Count on Plate
	4 days	6 days	7 days	10 days	14 days	
30 C	—	0	0	55.2	94.2	2,044
37 C	0	0.01	20.3	100	—	1,270

Basal medium prepared from dehydrated penassay broth and autoclave sterilized. Contained 10% horse serum and 10^{-2} M $MgSO_4$.

extract, 3%; sodium chloride, 7%; and distilled water for a total of 50 ml. To a 125-ml flask were added 10 ml of 2% glucose and either 10 ml of 7.36% K_2HPO_4 and 0 to 10 ml of 2.64% KH_2PO_4 or 10 ml of KH_2PO_4 solution and 0 to 10 ml of K_2HPO_4 solution. After autoclaving both flasks for 15 min at 121 C, the flasks were cooled and the required amount of separately sterilized buffer salt was added to the small flask to bring the total volume of each buffer salt solution to 10 ml (*see* Table 4). The contents (30 ml) of this flask were then added aseptically to the larger flask containing the other ingredients of the medium, yielding a total volume of 80 ml. To this was added the desired amount of potassium penicillin G (Pfizer), dissolved in sterile distilled water, sterile defibrinated horse serum (routinely 20 ml), and sterile 1 M $MgSO_4$ solution (routinely 2 ml). Finally, 100 ml of 1.6% agar at 50 C were added producing a total volume of 200 ml of the final agar medium which was then poured into plates and, after solidification, was inoculated as described above.

In cases when autoclave sterilization of glucose was not desired, 10 ml of each of the buffer salt solutions were added to the main flask before autoclaving and the glucose solution was filter sterilized and added aseptically after autoclave sterilization of the remainder of the medium. Tonicity of the medium was adjusted, when necessary, by the addition of the proper amount of sterile, 2 M sodium succinate solution (pH 7.0) to the autoclaved and cooled medium. In these cases and in others where serum amount was varied, the final volume of the completed medium was maintained at 200 ml by a corresponding reduction or increase in the volume of the agar solution.

RESULTS AND DISCUSSION

Conversion of 3A colonies to 3B colonies. Proposals of the cyclical nature of L form propagation, namely, conversion of 3A type L bodies to 3B type L bodies which, in turn, give rise to 3A type L units, have been advanced by Dienes and others (reviewed in Dienes and Weinberger (1951); Liebermeister (1960)). Evidence for conversion of 3A colonies to 3B colonies was sought in strain F of Proteus.

Plates of medium II (agar, 0.8%; pencillin, 200 units; succinate, none) were inoculated with cells of Proteus strain F and incubated at 30 C and at 37 C. Total and differential L colony counts were performed as soon as the colonies became visible and were repeated at intervals until conversion was complete. Typical results from such an experiment are presented in Table 1. The total L colony count remained the same throughout the period of incubation. It is apparent that conversion of 3A colonies to 3B colonies begins earlier and proceeds more rapidly, at 37 C than at 30 C. Clearly, the yield of morphologically distinct 3A type colonies from a given inoculum of Proteus cells will depend on the time and temperature of incubation on medium II.

Detection of an inhibitor of 3B type L growth. Adoption of a constant time and temperature of incubation, namely 6 days at 30 C, failed to yield reproducible data and the percentage of 3B type or 3A type L colonies obtained in apparently identical experiments varied from less than 1.0% to greater than 50%. A consideration of this inconsistency, coupled with the phenomenon of colony conversion documented above, suggested that in the freshly prepared medium there was a substance which suppressed 3B type L growth but which lost its activity during incubation. It followed that uninoculated but aged medium should have lost this inhibitor and would then be capable of supporting the 3B type of L growth. Thus, several flasks of double strength penassay broth were prepared and sterilized by autoclaving. To one series of flasks, double strength agar (1.6%) was added and the medium was poured into plates. These plates were then inoculated with Proteus strain F cells and incubated for 6 days at 30 C. The other series of flasks was aged for 7 days at 37 C, following which double strength agar was added and the medium poured into plates. These plates

TABLE 2

Comparison of freshly prepared and aged medium II for growth of 3B type L colonies of Proteus mirabilis

Plating Medium Used	Total L Colony Count	Per Cent 3A Types
Freshly prepared	4.8×10^7	100
Aged for 7 days at 37 C	1.64×10^8	<0.05

Basal medium prepared from dehydrated penassay broth (Difco) and autoclaved sterilized. Contained 10% horse serum and 10^{-2} M $MgSO_4$.

were then inoculated and incubated in a manner identical to that described above. The results of such an experiment are presented in Table 2. These data clearly demonstrate that freshly prepared medium strongly inhibits growth of 3B type L colonies and that aged medium supports relatively luxuriant growth of this L colony type. Further experiments of this general type provided conclusive evidence that the inhibitory substance was present in the penassay medium itself and that horse serum played no direct role in the inhibition noted. Additionally, it was found that filter sterilized medium supported 3B type L growth at least as well as aged, autoclaved sterilized medium.

Origin of the 3B inhibitor. An exploration of the behavior of the individual ingredients of penassay medium was conducted to detect the component(s) responsible for the production of the inhibitor of 3B type L growth. Concentrated stock solutions of the ingredients of penassay broth were prepared and, from these solutions, various flasks of media were formulated, each flask lacking in a single component of the complete medium. Such deficient media were sterilized by autoclaving, following which the missing ingredient was filter-sterilized and added aspetically. An equal volume of double strength agar solution was added, the medium poured into plates, and inoculated and incubated as previously described. The results of these experiments showed that only the medium in which glucose was omitted during autoclave sterilization was devoid of the inhibitor of 3B type L forms. Further trials demonstrated that when glucose was autoclaved alone, no 3B inhibitor arose, indicating that there was some interaction between glucose and another medium ingredient which yielded 3B inhibitor upon autoclaving.

TABLE 3

Production of 3B inhibitor during autoclaving of glucose with various components of penassay broth

Glucose Autoclaved with	Total Volume During Autoclaving	L Colony Count per Ml	
		3A Type	3B Type
	ml		
Buffer salts	30	2.2×10^7	5.2×10^6
NaCl	20	3×10^6	9.4×10^5
Beef extract	20	1×10^7	7.7×10^8
Yeast extract	20	3×10^6	9.3×10^8
Peptone	20	5×10^6	9.3×10^8
All ingredients filter sterilized	—	5×10^6	1.1×10^9
All ingredients mixed and autoclave sterilized	80	2.1×10^7	2.6×10^8

Media prepared from individual ingredients of penassay broth. All media contained 10% horse serum, 10^{-2} M $MgSO_4$, 0.8% agar, and 200 units of penicillin G per ml.

As a consequence of the observations presented above, glucose was autoclaved with various single medium components and then added to the remainder of the ingredients which had been autoclaved together. The data of Table 3 present these results. It is clear that autoclave sterilization of glucose produced the inhibitor only in the presence of the buffer salts employed in the complete medium. A survey of various other carbohydrates revealed that this reaction was nonspecific and that the 3B inhibitor was produced when any one of several reducing carbohydrates was autoclaved with the phosphate buffer salts. Sucrose, several hexitols, and glycerol did not yield the inhibitor when autoclaved in the presence of the buffer.

An assessment of the function of pH in the generation of the inhibitor offered a clue as to the probable chemical nature of the substance. Glucose was autoclaved with various mixtures of the phosphate salts to produce differing pH values after which the remaining amount of the particular buffer salt was added. The resulting glucose-phosphate mixtures were added to the other medium constituents, and from this preparation the soft agar medium was produced and inoculated as previously described. Only

TABLE 4

Production of 3B inhibitor during autoclave sterilization of glucose in phosphate buffer of varying pH

Ml per Flask of				pH Before Autoclaving	L Colony Count per Ml	
Water	2% Glucose	7.36% K_2HPO_4	2.64% KH_2PO_4		3A Type	3B Type
10	10	0	10	4.4	5.6×10^7	1.64×10^8
10	10	2	10	6.4	1.0×10^8	9.9×10^7
10	10	5	10	6.8	1.05×10^8	2.4×10^7
10	10	10	10	7.1	1.03×10^8	4×10^4
10	10	10	5	7.3	1.03×10^8	5×10^4
10	10	10	2	7.7	8.2×10^7	4×10^4
10	10	10	0	8.8	6.8×10^7	4×10^4

Media prepared from individual comparison of penassay broth. All media contained 10% horse serum, 10^{-2} M $MgSO_4$, 0.8% agar, and 200 units of penicillin G per ml.

at alkaline pH values was there an appreciable generation of the 3B inhibitor (Table 4) and there was a striking difference in yield of inhibitor between pH 7.1 and pH 6.8. Potentiometric measurements showed that after autoclaving at alkaline pH values, the pH of the glucose-phosphate mixture had dropped to approximately pH 6.8, whereas the reaction of glucose-phosphate mixtures autoclaved at acid pH values was unaltered. Approximately 3 mmoles of mineral acid are required to reduce the pH 8.8 glucose-phosphate solution to pH 6.8. Therefore, nearly 3.0 mmoles of acid had been produced from 1.0 mmole of glucose, (10 ml of 2% solution) indicating extensive destruction of the glucose molecule. Alkaline oxidation of sugars, catalysed by phosphate, is known and described in the literature (Pigman and Goepp, 1948), and yields a mixture of low molecular weight organic acids among which lactic, acetic, formic, and oxalic are predominant. The fact that the 3B inhibitor produced by autoclaving glucose-phosphate mixtures at alkaline pH lost activity upon storage at 37 C pointed strongly to relatively volatile formic acid as the active substance.

The organic acids resulting from alkaline oxidation of glucose were tested singly in media which were prepared with filter sterilized glucose. Simultaneously, duplicate series of media containing one of these acids were prepared and allowed to age for 7 days at 37 C. The 3B inhibitory properties of all such freshly prepared and aged media were assessed by the methods already described. The results (Fig. 1) demonstrate that formic, oxalic, and acetic acids all strongly suppress growth of 3B type L colonies but that lactic acid has no inhibitory activity and could be dismissed from consideration. Furthermore, only media to which formic acid was added lost the ability to suppress 3B type L growth upon 37 C aging. As a result of these experiments, the major 3B inhibitor generated during autoclave sterilization of glucose-phosphate mixtures has been tentatively identified as formic acid.

Antagonism of 3B inhibitor by toxicity. The precise mode of action of formic acid is, as yet, unknown. The view was adopted that the 3B inhibitor acted as a block in cell wall synthesis and as such, in conjunction with penicillin, permitted the cell to produce only a very tenuous membrane which could not maintain its physical integrity on medium without the stabilizing effect of hypertonicity. This hypothesis was tested by determining the growth of 3B type L colonies on media containing specified amounts of the 3B inhibitor and varying amounts of sodium succinate as tonic support. When such media were inoculated with cells of Proteus strain F, the numbers of 3A type and 3B type L colonies obtained are those presented in Table 5. As the amount of 3B inhibitor in the medium is increased, there is a corresponding increase in the amount of sodium succinate required to yield the maximal number of 3B type L colonies. At relatively high concentrations of inhibitor, there is a narrow range of tonicity in which the growth of both the 3A type and 3B type L colonies is suppressed. No explanation can be offered for this phenomenon at present. Additional experiments have demonstrated that the

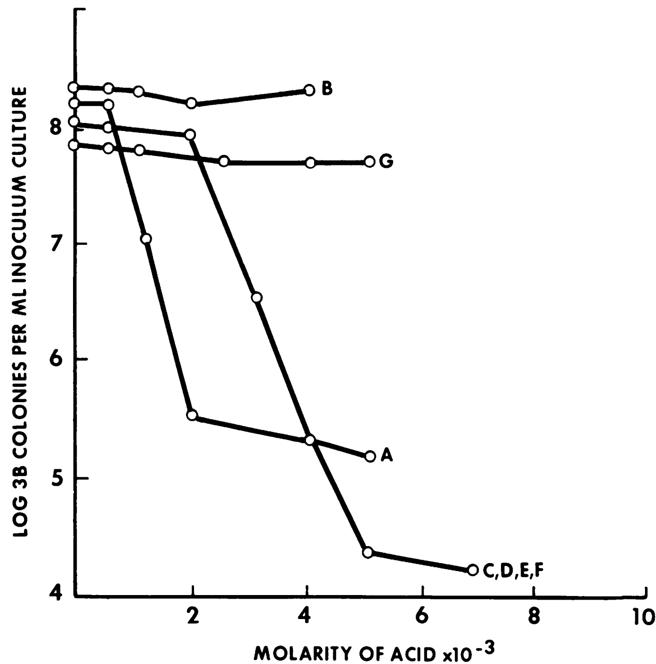

Fig. 1. The 3B inhibitory activity of freshly prepared and aged media containing low molecular weight organic acids. Media prepared from individual ingredients of penassay broth. Glucose sterilized by filtration. All media contained 10% horse serum, 10^{-2} M $MgSO_4$, 0.8% agar, and 200 units of penicillin G per ml. *A*) Formic acid medium freshly prepared; *B*) aged formic acid medium; *C*) acetic acid medium freshly prepared; *D*) aged acetic acid medium; *E*) oxalic acid medium freshly prepared; *F*) aged oxalic acid medium; *G*) lactic acid medium freshly prepared.

TABLE 5

Effect of sodium succinate on inhibition of 3B type L growth by autoclaved glucose phosphate solutions

Molarity of Sodium Succinate	3B Type Colony Count per Ml with Glucose Autoclaved at pH of			3A Type Colony Count per Ml with Glucose Autoclaved at a pH of		
	7.1	7.7	8.8	7.1	7.7	8.8
0	2×10^6	1×10^6	$<10^6$	3.2×10^7	1.6×10^7	1.3×10^7
0.05	1×10^6	$<10^6$	$<10^6$	1.4×10^7	1.3×10^7	9×10^6
0.1	2×10^6	$<10^6$	$<10^6$	7×10^6	5×10^6	$<10^6$
0.2	8.4×10^7	2.6×10^7	2.4×10^7	2×10^6	1×10^6	$<10^6$
0.3	6.8×10^8	4.2×10^8	2.7×10^8	2×10^6	$<10^6$	$<10^6$

Media prepared from individual ingredients of penassay broth. All media contained 10% horse serum, 10^{-2} M $MgSO_4$, 0.8% agar, and 200 units of penicillin G per ml.

3B inhibitory activity of acetic and oxalic acids is also overcome by increases in tonicity of the medium.

Reversal of 3B inhibition by penicillin. Preliminary qualitative experiments, cited in the introduction, indicated that low concentrations of penicillin (100 to 200 units per ml) yielded the maximal number of 3A type L colonies from an inoculum of Proteus cells. The established facts of conversion of 3A type to 3B type L colonies upon prolonged incubation and the existence of an active inhibitor of 3B type L colonies necessitated a reassessment of the effect of penicillin concentration on the yield of L colonies from a cell inoculum. The plating medium was prepared containing varying amounts of 3B inhibitor and

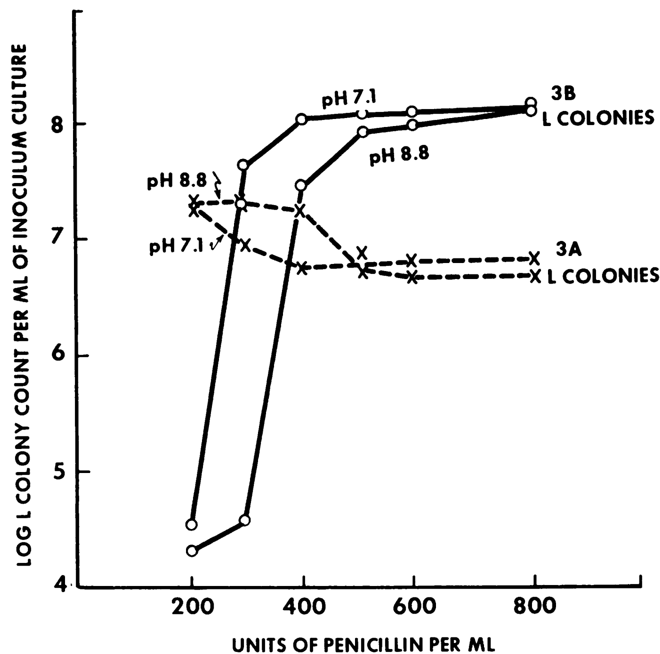

Fig. 2. Reversal by penicillin of the 3B inhibition by autoclaved glucose-prosphate solutions. Media prepared from individual ingredients of penassay broth. Glucose-phosphate mixtures autoclaved at indicated pH values and added to other medium ingredients as described in methods section. All media contained 10% horse serum, 10 M^{-2} $MgSO_4$, and 0.8% agar.

penicillin and such media, when inoculated with cells of Proteus strain F, allowed L colony growth as depicted in Fig. 2. These unexpected results showed a striking effect of penicillin concentration on the number of 3B type L colonies obtained. There is apparently a sharply defined titration of the 3B inhibitor by the penicillin molecule which renders the 3B inhibitor inactive. This effect cannot be attributed to tonicity increase from the higher penicillin concentrations which modify the tonicity very slightly. The ring nitrogens of the penicillin molecule exhibit no basicity and therefore cannot form a salt with the 3B inhibitor. However, the penicillin retains the property of inhibition of cell wall formation since antagonism of 3B inhibitor by succinate yields 3B type L colonies in the presence of low penicillin concentrations. Penicillin also completely neutralizes the 3B inhibitory action of acetic and oxalic acids.

These results are consistent with the assumption that formic, acetic, and oxalic acids attach to the surface of the spheroplast and induce a leaky condition with resulting lethality. High tonicity prevents leakage of essential compounds from the spheroplast by osmotic effects, whereas penicillin may displace formate, oxalate, or acetate from the sites of attachment, thus maintaining the selective permeability and viability of the spheroplast.

Nature of serum requirement. Several views of the role of serum in media for the growth of L forms are recorded in the literature. Dienes and Weinberger (1951) have stated that there is an absolute requirement for serum for 3A type L growth whereas Medill and O'Kane (1954) in a study of 3B type L growth of Proteus attribute the need for serum to detoxification of inhibitory components of the medium. Therefore, the effects of variation in amounts of serum on the yield of both types of L colonies obtained from an inoculum of Proteus cells were determined. The basal medium consisted of autoclave sterilized penassay broth and contained a small but effective amount of 3B inhibitor. The data presented in Fig. 3 show clearly that serum is indeed necessary for 3A type growth and that serum is not required for growth of 3B type L colonies. Furthermore, relatively high amounts of serum increase the yield of 3B type L colonies, probably

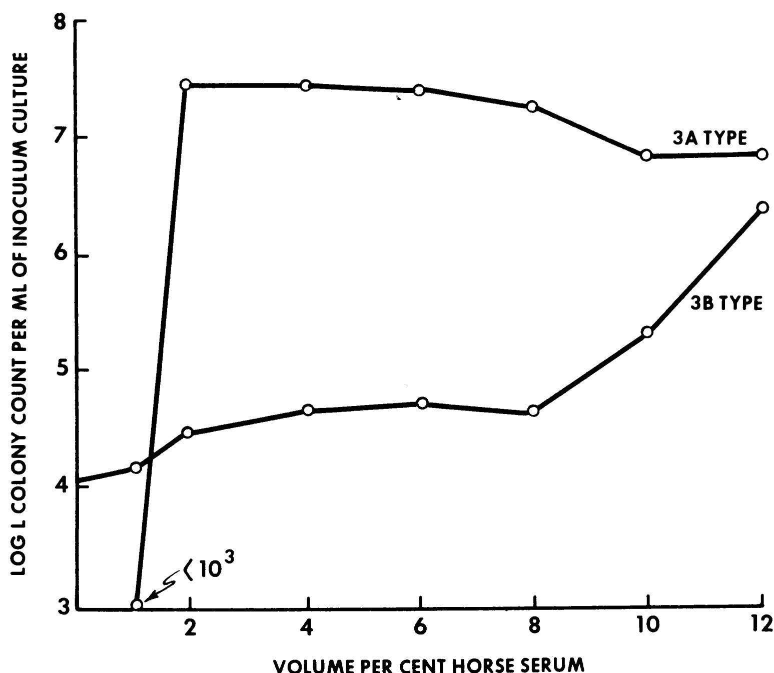

Fig. 3. Variation in yield of 3A and 3B type L colonies as a function of concentration of horse serum. All media prepared from dehydrated penassay broth (Difco), sterilized by autoclaving, and contained 10^{-2} M $MgSO_4$, 0.8% agar, and 200 units of penicillin G per ml.

by binding the 3B inhibitor. Serum samples which have been heat inactivated by exposure to 56 C for up to 1 hr gave identical results, thus minimizing a possible role of complement-mediated immunological reactions leading to lysis of the cell membrane or wall. Exhaustive dialysis of serum against distilled water failed to alter either the promotion of 3A type growth from low serum amount or the support of 3B type growth from high serum concentration. It appears as if both functions of horse serum are carried out by the macromolecular components.

Yield of 3A type L colonies from various strains of Proteus. Other isolates of *P. mirabilis* were evaluated to determine if the factors found to influence strain F also controlled production of L forms from randomly selected strains. Bacillary cells of these strains were plated on medium II containing varying amounts of 3B inhibitor. The data in Table 6 reveal that there was marked suppression of 3B type L colonies with any strain but that the amount of 3B inhibitor and the period of incubation were of importance in permitting the expression of the maximal yield of 3A type L colonies. The maximal number of 3A type L colonies resulting from inoculation with a constant number of bacillary cells varied widely, depending upon the strain employed.

Effect of medium composition on yield of 3A type L colonies. In the experiments reported above, the number of 3A type L colonies resulting from a given inoculum of cells of a specific strain remained relatively constant in contrast to wide variation in yield of 3B type colonies. The cell to L ratio (Altenbern and Landman, 1960) for the 3A type L colony was in the range of 500 to 600 for strain F. There were indications that some modifications of the medium resulted in a real change in the number of 3A type L colonies obtained. Small amounts of 3B inhibitor permitted greater numbers of 3A colonies to develop (Table 4), and lesser amounts of serum yielded a similar result (Fig. 3). Alteration in concentration of magnesium sulfate had a striking effect upon the cell to L ratio for the 3A type L colony. The data in Table 7 show that high concentrations of $MgSO_4$ increased significantly the total number of 3A colonies obtained from a constant inoculum of cells. Additional changes in medium composition showed that an improved yield of

TABLE 6

3A Type L colony formation from various strains of Proteus mirabilis plated on medium II (agar) containing 3B inhibitor

Glucose-Phosphate Autoclaved at pH	Proteus Strain	3A Type L Colony Count after Incubation at 30 C for:	
		6 Days	12 Days
7.1	A	1.4×10^6	3.7×10^6
7.1	B	$<10^3$	1.3×10^6
7.1	E	8×10^3	3.8×10^5
7.7	A	1.59×10^6	4.4×10^6
7.7	B	$<10^3$	7.0×10^5
7.7	E	3×10^3	2.2×10^5
8.8	A	$<10^3$	$<10^3$
8.8	B	$<10^3$	$<10^3$
8.8	E	$<10^3$	$<10^3$

Media prepared from individual ingredients of penassay broth. All media contained 10% horse serum, 10^{-2} M $MgSO_4$, 0.8% agar, and 200 units of penicillin G per ml.

TABLE 7

Effect of $MgSO_4$ concentration on yield of 3A type L colonies from Proteus F cells plated on medium II containing 3B inhibitor

Glucose-Phosphate Autoclaved at pH	Final Molarity of $MgSO_4$	3A Type L Colony Count	Cell to L Ratio
7.1	0.005	1.4×10^7	593
7.1	0.01	2.0×10^7	430
7.1	0.02	3.4×10^7	253
7.1	0.04	5.3×10^7	162
7.7	0.005	7.8×10^6	1,100
7.7	0.01	1.4×10^7	614
7.7	0.02	1.9×10^7	452
7.7	0.04	3.4×10^7	253

Medium prepared from individual ingredients of penassay broth. All media contained 10% horse serum, 0.8% agar, and 200 units of penicillin G per ml.

3A colonies was obtained on media containing one-tenth the standard amount of peptone or yeast extract. With a combination of low serum concentration (4% v/v), low concentration of 3B inhibitor, reduced peptone and yeast extract content, and 0.02 to 0.04 M $MgSO_4$, a cell to L ratio of 100 for the 3A colony type could be obtained. Variations in agar concentration from 0.6 to 1.2% were without effect and four separate lots of agar were indistinguishable with regard to promotion of 3A type L growth.

Numerous changes in composition of the medium for cultivating the inoculum cells did not alter the yield of 3A colonies obtained on a solid medium of constant composition. However, reduction in incubation temperature for growth of the inoculum from 37 C to 23 C reduced the cells to L ratio for 3A L colonies by 40%. A twofold reduction in cell to L ratio was obtained by employing inoculum cells grown for 96 hr at 37 C vs. 24 hr at 37 C. By combining the optima determined for these single variables, a cell to L ratio for 3A type L forms of 68 could be obtained.

As in the case of 3B type L colonies (Landman et al., 1958), there was uncertainty as to whether 3A type L colony formation resulted from a small proportion of suitable genotypes in the cell population or whether all cells of the population could form 3A colonies under optimal conditions. Although a cell to L ratio of 1.0 has not been realized, as has been obtained for the 3B colonies, it has been demonstrated that the cell to L ratio for 3A colonies may be varied from 1,100 or more to 68 by changes in medium composition or growth of inoculum cells. Furthermore, an isolate of Proteus bacilli, obtained from reversion of a 3A colony to the bacilliform state on penicillin-free medium, yielded the same cell to L ratio for 3A colonies as did the parent stock culture cells. These observations seem to establish that the ability of a Proteus cell to form 3A type L growth on suitable medium is not a genetic property of a few cells in the population but is the result of a physiological process which, theoretically, can be completed by all viable cells of a suitable inoculum on a medium of the proper characteristics. However, the complete suppression of growth of 3B type colonies by formate or the other small organic acids, which are without pronounced effect on growth of 3A colonies, suggests that those cells of an inoculum, which are destined to produce 3A type L colonies, do not initially pass through a large body (3B) state that would be inhibited by formate. In contrast, reversion of 3A type L forms to bacilli on penicillin free medium is very strongly suppressed by 3B inhibitor (Altenbern, *unpublished data*, 1959) and suggests that the 3B form is intermediate in the reversion of the 3A type of L forms to bacilli.

Many pictorial presentations of 3A L colony

morphology in the literature show a considerable size range of particles, from very tiny granules to large, vacuolated, and apparently empty spheres. Addition of the proper amount of 3B inhibitor (formate) to medium II seems to purify the 3A colonies growing thereon and only small, subcellular granules can be seen by methylene blue staining or by phase contrast microscopy. On media possessing marginal amounts of 3B inhibitor (suppression of 50% of the total, potential 3B colonies), 3A type L colonies do possess elements varying greatly in size. However, there is no reason to believe that increase in size of the elements is a necessary condition for propagation of 3A type L forms and indeed it appears that the presence of large elements in 3A colonies is secondary and unrelated to the basic mode of propagation of 3A type L particles. Unpublished experiments in this laboratory with suspensions of 3A particles from such "purified" colonies clearly indicate that the 3A type L form is a distinct state, separable from bacilli and 3B type L forms by a number of criteria.

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SUMMARY

The 3A type L form of *Proteus mirabilis* gives rise to the 3B type L form during prolonged incubation, and this conversion of colony morphology is a function of both time and temperature of incubation. During autoclaving of complete medium, a substance inhibiting the growth of 3B type L forms from an inoculum of cells is produced. Analysis of the interaction of various components of the medium showed that the 3B inhibitor is generated during autoclave sterilization of glucose at alkaline pH in the presence of phosphate buffer salts. On the basis of reconstruction experiments, this inhibitor has been tentatively identified as formic acid. Both hypertonicity produced by 0.3 M sodium succinate and high penicillin concentrations effectively antagonized the action of the 3B inhibitor. The role of horse serum in promoting growth of both types of L forms has been examined. Some effects of medium composition on the total yield of 3A type L colonies from a given inoculum of cells are presented.

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