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Defined Physiological Conditions for the Induction of the L-Form of *Neisseria gonorrhoeae*

BARBARA A. BACIGALUPI AND JOHN W. LAWSON
School of Public Health, University of California, Berkeley, California 94720

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Defined conditions are described which allowed luxuriant growth over continuous subculture of strains of *Neisseria gonorrhoeae* in broth and on agar. Growth was equal to or surpassed that observed in Mueller-Hinton broth or on Mueller-Hinton blood agar. The final medium adopted consisted of medium 199 and a supplemental mixture of cysteine, glucose, and various salts. Addition of sodium bicarbonate or CO_2 enrichment was not required. For solidification, only agarose allowed growth of all strains; glutamic acid stimulated growth of two strains but was inhibitory for a third. The addition of 8% polyvinylpyrrolidone (PVP), 2% purified albumin, and penicillin resulted in induction of all three strains to the L-form with frequencies up tọ 0.3%. At present no induction to the L-form has been achieved in the absence of albumin. Various lots of PVP proved toxic in the defined medium, and extensive dialysis was required for good growth and L-form induction. Substitution of PVP with sucrose indicated a sucrose toxicity for the parental gonococcus even on the addition of albumin. L-form induction did occur on sucrose L-medium but at significantly lower frequencies. The colonies appeared 1 week later than those on PVP L-medium but at significantly lower frequencies. The colonies appeared 1 week later than those on PVP L-medium and remained very small and poorly developed.

As early as 1959, the suggestion was made implicating the L-form of *Neisseria gonorrhoeae* in penicillin treatment failures of gonorrhea (2). Recently, Gnarpe, Wallin, and Forsgren (7) reported the successful isolation of the gonococcal L-form from 12% of the clinical specimens taken from patients with gonorrhea. Despite negative cultures by conventional methods, in 4.5% of the cases the bacterial parent or L-form could only be isolated on osmotically stabilized medium. The possible pathogenic role of wall-defective variants in chronic infections (4) has brought renewed interest in definition of the physiological conditions which permit induction and persistence of the L-form, as well as the elucidation of its biochemical and antigenic properties.

The in vitro induction systems reported thus far have indicated tremendous variation in inducibility and propagation of the L-form, dependent on several cultural conditions, including the osmotic stabilizer used. Although NaCl would allow the isolation of the gonococcal L-form, Dienes, Bandur, and Madoff (5) were unsuccessful in all propagation attempts. Roberts (11) also achieved low levels of L-form induction; however, his sucrose-stabilized medium did support growth upon subculture. Lawson and Douglas (Can. J. Microbiol., in press) have recently demonstrated that the incorporation of polyvinylpyrrolidone (PVP) into the induction medium resulted in massive (greater than 10%) conversion to the L-form of all tested strains of *N. gonorrhoeae*, including seven strains which were shown to be noninducible on sucrose-stabilized medium.

Defined cultural conditions will allow assessment of the physiological variables functioning in L-form induction, growth, and reversion. The present paper describes the adaption of three strains of *N. gonorrhoeae* to growth in synthetic media, both fluid and agar. Patterns of growth on solid chemically defined medium were correlated with the type of agar employed for solidification. Induction to the L-form was achieved by the incorporation of dialyzed polyvinylpyrrolidone and albumin into the solid synthetic medium in the presence of penicillin.

MATERIALS AND METHODS

Bacterial strains. Cultures of *N. gonorrhoeae*, strains 474 (a gential isolate), 448 (a throat isolate), and GC#3 (a fresh clinical isolate) were provided by the Neisseria Repository, Naval Medical Research Unit #1, University of California, Berkeley. All strains were confirmed as *N. gonorrhoeae* by Gram stain, positive oxidase reaction, fermentation with glucose, but not with maltose, sucrose, mannitol, lactose, or fructose, and agglutination with gonococcal antisera.

Media. The formulation for the synthetic fluid medium, which shall be referred to as GC-SM broth, is described in Results. Medium 199–10× with Hanks salts and glutamine, without sodium bicarbonate, was a product of Grand Island Biological Co., Grand Island, N. Y. The amino acids and β-glycerophosphate, sodium salt, were obtained from Calbiochem, Los Angeles, Calif. Inorganic chemicals were reagent grade (Mallinkrodt Chemical Works, St. Louis, Mo., or Allied Chemical Company, Morristown, N.J.). Membrane filters were obtained from the Millipore Corp., Bedford, Mass.; pore sizes have been indicated in parentheses. The chemically defined medium base was filter sterilized (0.45 μm) after adjustment of pH to 7.0 with 2 N NaOH. Water used in the preparation of all media was distilled and then deionized. All glassware was washed to tissue culture specifications. Solid synthetic medium was achieved by the addition of 1% purified agar (Difco, Detroit, Mich.), 1% Ionagar no. 2 (Consolidated Laboratories, Chicago Heights, Ill.), or 1.5% agarose (Bio-Rad Laboratories, Richmond, Calif.) to the GC-SM base. Under certain circumstances, the glutamic acid concentration was increased by supplementing the solid synthetic medium with 1.46 g of DL-glutamic acid per liter. Pharmaceutical grade PVP, average molecular weight of 40,000 (Sigma Chemical Co., St. Louis, Mo.), was prepared for incorporation into the defined induction medium by dialysis of a 30% solution at 4 C for at least 48 h. The final concentration, measured by the method of Ashwood-Smith and Warby (1), was approximately 7.0%. The GC-SM base components were added directly to the PVP solution which had been prefiltered (0.8 μm) after dialysis and supplemented with 2% bovine albumin (fraction V from bovine plasma, Reheis Chemical Company, Chicago, Ill.). After pH adjustment and filter sterilization, the base medium was combined with an autoclaved solution of 10× Ionagar. Sucrose-stabilized, defined induction medium was obtained by substitution of the dialyzed PVP with 10% sucrose. A 5-ml amount of media was poured into petri dishes (60 by 15 mm, Falcon, Oxnard, Calif.). One hour prior to plating 50 U of potassium penicillin G (Squibb) was spread over the surface of each plate, with a resulting, final estimated concentration of 10 U of penicillin per ml. These two induction media will be referred to as PVP L-medium or sucrose L-medium. Complex induction medium was prepared by the specifications previously described (J. W. Lawson and J. T. Douglas, Can. J. Microbiol, in press).

Maintenance and storage of the organism. Stock cultures of all strains were maintained in both complex and defined media. GC-SM broth cultures were subcultured daily and incubated at 37 C. Once the organisms were adapted to the synthetic medium, the inoculum size was decreased in a step-wise fashion from 10 to 1%. All solid media plates were incubated at 37 C in a sealed chamber flushed with a gaseous mixture of 10% CO_2 in air. For storage of strains adapted to GC-SM, 0.1-ml samples of 24-h broth cultures were lyophilized. Such lyophilized cultures were revived by plating a sample of rehydrated cells in solid GC-SM supplemented with 2% albumin. After 24 h of incubation, growth was harvested, inoculated into GC-SM broth without albumin, and subcultured for several days prior to use.

Viable counts. Growth in GC-SM broth was followed by plating appropriate dilutions on Mueller-Hinton blood agar plates (MH-BAP) and determining the number of colony-forming units (CFU) per ml.

L-form induction procedures. The induction of the gonococcal L-form in complex medium was performed as previously described (J. W. Lawson and J. T. Douglas, Can. J. Microbiol., in press). For induction in the defined system, appropriate dilutions of a 12-h culture grown in GC-SM broth were plated on defined L-media. The number of parental gonococci was determined by plating samples from culture dilutions made in GC-SM broth on both L-media without penicillin and MH-BAP. Incubation of L-media was continued for 10 to 14 days in the CO_2-enriched chamber at 37 C. Induction frequency was expressed as the percentage of L-colonies scored on L-media as compared with the total number of parental gonococci isolated on the same media without penicillin.

Light microscopy. Photographs of parental and L-form gonococci were obtained with a Pentax Spotmatic camera mounted on a light microscope. Wetmount preparations were stained by the Dienes method prior to photographing.

RESULTS

Growth in synthetic medium. Initially, strains 474 and 448, but not GC#3, were successfully adapted from Mueller-Hinton Broth cultures to growth in synthetic fluid medium (Table 1) to which 1.3 g of D-glutamic acid per liter had been added. None of the three strains were capable of growth in either medium 199 (final concentration 1.0× or 0.5×) or the supplemental salts, glucose, and cysteine alone. Both strains 474 and 448 produced turbid growth in GC-SM with all three concentrations of medium 199 tested; 1.0×, 0.5×, and 0.25×. Since 0.5× concentration of the tissue culture medium produced optimum 24-h viable counts of approximately 2×10^8 CFU/ml, the remaining two concentrations were discontinued for further studies.

A modification of the supplements initially added to medium 199 was sought which would expand the growth-supportive capacity of the GC-SM broth to include strain GC#3. A series

TABLE 1. *Synthetic medium GC-SM[a, b] for the growth of Neisseria gonorrhoeae*

Substance	Amount/liter
Medium 199-10X	50.0 ml
1-Cysteine-HCl	0.012 g
Na-glycerophosphate	2.5 g
KCl	0.09 g
NaCl	6.0 g
NH_4Cl	1.25 g
$MgSO_4 \cdot 7H_2O$	0.6 g
Glucose	5.0 g

[a] Final pH was adjusted to 7.0 with 2 N NaOH.
[b] For solid medium 15.0 g of agarose was added per liter.

of media was tested, each lacking one of the supplemental components. Strains 474 and 448 were easily adapted, i.e., subcultured beyond 10 passes, to seven out of eight media, whereas strain GC#3 achieved growth only if glutamic acid was excluded. On the basis of this data, glutamic acid was no longer included in the GC-SM broth. Further studies are in progress to determine the minimum supplements necessary for growth. If the sodium glycerophosphate was omitted, adaption was not as successful for strains 474 and 448. Although medium 199 is buffered, an additional buffering system seemed to be required. For medium lacking the additional buffer, the final pH of 24-h-old cultures was 5.75 compared to all other media tested, which remained approximately 6.3. Growth in fluid GC-SM was not dependent on the addition of sodium bicarbonate to the medium 199.

Although throughout the adaption period inocula size was maintained at 10%, eventually all three strains were successfully reduced to a 1% subculture. Increased CO_2 tension, shaking, or tightly fitted caps were not required for the growth of fluid cultures. Cultures usually became turbid 6 to 8 h after inoculation, and the phenol red indicator color change to yellow most often occurred between 8 to 10 h. Figure 1 shows a graphical expression of typical growth for strain 474 in GC-SM broth. After a short lag phase, exponential growth proceeds for approximately 5 h with a mean doubling time of 43 min. Viable counts consistently remain about 10^8 CFU/ml for nearly 24 h, followed by a rapid drop as death phase ensues. Subcultures of 48-h-old cultures are only rarely successful.

During the adaption period, the cultures appeared more granular than the even turbidity characteristic of well-adapted cultures. Light microscope examinations of low-passage cultures revealed clusters, tetrads, and chains of gonococci as well as the usual diplococcal forms which predominated in highly adapted cultures. Morphological changes could be associated with the stationary phase of growth. The cocci became swollen, and large bodies ranging in size up to 20 μm appeared. When using the Dienes staining methods, both the swollen cocci and large bodies were Dienes positive (Fig. 2). Adapted cultures lost their tendency to produce both of these morphological changes. Large bodies were routinely observed in all low-passage cultures of strains 474 and 448 in synthetic media, regardless of which of the supplemental components had been omitted. Gram stains consistently showed gram-negative, swollen cocci as well as round patches of gram-negative debris comparable in size to the large bodies observed in wet-mount preparations.

Growth on solid synthetic medium. Preliminary experiments utilized the incorporation of 1% purified agar into the GC-SM base. By the third passage, both strains 474 and 448 exhibited very poor growth on the solid matrix. Strain GC#3 was incapable of growth on this medium. Increasing the glutamic acid concentration by supplementing the solid GC-SM with an additional 1.46 g of DL-glutamic acid per liter resulted in luxuriant growth (more than 10^8 CFU/ml) and successful subculture for only

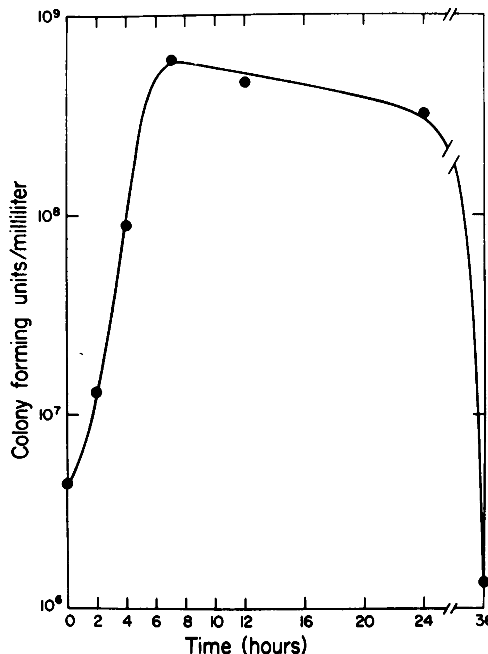

FIG. 1. *Growth of N. gonorrhoeae, strain 474 in synthetic medium. A 1% inoculum from a 12-h culture grown in fluid GC-SM was added to fresh GC-SM and incubated at 37 C.*

strain 448. To extend the growth-supportive capacity of the medium, both 1% Ionagar no. 2 and 1.5% agarose were tested as substitutes for the purified agar. The comparison between these three agars is summarized in Table 2. Strains 474 and 448 both produced good initial growth on GC-SM solidified with Ionagar no. 2, but upon passage, strain 474 showed only a thin film of growth over the agar surface. This sparse growth was greatly enhanced by the addition of supplemental DL-glutamic acid. Again strain GC#3 did not grow on the Ionagar medium unless 2% albumin was added. Only the utilization of agarose in the solid GC-SM allowed the growth of strain GC#3, in addition to the luxuriant growth of strains 474 and 448. Supplemental glutamic acid seemed to have no effect on the growth of strains 474 or 448 on agarose solidified medium but was inhibitory for strain GC#3. Agarose-solidified GC-SM was prepared with three different medium 199 concentrations; 1.0×, 0.5×, and 0.25×. More than 10^8 well-developed colonies with strains 474 and 448 were recovered at all the concentrations tested. In contrast, strain GC#3 exhibited no growth on 1.0× concentration and poor growth on 0.25× medium 199, compared to the satisfactory growth noted on 0.5× concentration, which could be easily passed.

Incubation of the plates at 37 C in a sealed chamber flushed with a gaseous mixture of 10% CO_2 and 90% air resulted in the appearance of colonies on the solid GC-SM within 18 to 24 h. Growth of strain GC#3 tended to be slower; colonies were usually visible after 30 h. Typical gonococcal colony morphology was observed on this medium, as exemplified by strain 474 in Fig. 3. Analogous to the findings in fluid cultures, subcultures to either broth or solid GC-SM of 48-h-old colonial growth were seldom successful.

L-form induction in defined medium. Massive induction to the L-form on complex PVP L-medium was established for all three strains prior to their introduction into defined cultural conditions. After adaption to growth in fluid GC-SM, their inducibility was in no way diminished. For purposes of screening the solid defined medium's capability for supporting induction to the L-form, only strain 474 was utilized in the preliminary studies. In the presence of penicillin, the incorporation of 8% untreated PVP and 2% albumin resulted in L-form induc-

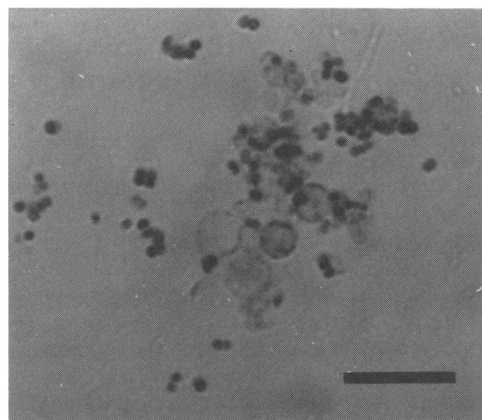

FIG. 2. *GC-SM fluid culture (24 h) of gonococcal strain 474. Cells stained by the Dienes method. ×970. Bar, 5 μm.*

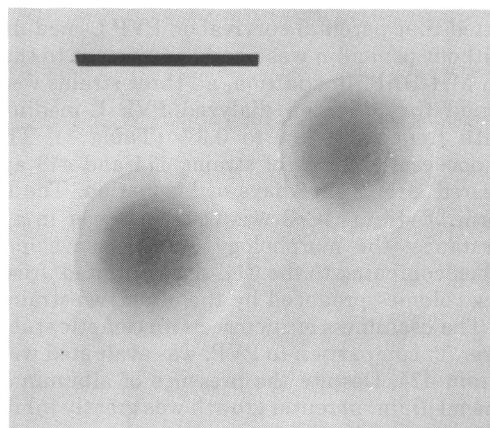

FIG. 3. *Parental colony (48 h) of gonococcal strain 474 grown on GC-SM. ×80. Bar, 0.1 mm.*

TABLE 2. *Effects of various agars on the supplemental glutamic acid[a] requirements for growth[b] of N. gonorrhoeae in GC-SM*

Strain	– Glutamic acid			+ Glutamic acid		
	Purified agar	Ionagar no. 2	Agarose	Purified agar	Ionagar no. 2	Agarose
474	±	±	+	–	+	+
448	±	+	+	+	+	+
GC#3	–	–	+	–	–	–

[a] 1.46 g of DL-glutamic acid per liter.

[b] Symbols: +, luxuriant growth after several subcultures, ±, little or no growth on subculture, –, no growth.

tion, characterized by the typical fried-egg morphology depicted in Fig. 4. Highly vacuolated large bodies were observed in the peripheral zone of the L-colonies when stained by the Dienes method. The dense, granular central areas of the colonies appeared to penetrate into the agar matrix in the typical fashion. Unpublished data further indicated that colonies of similar morphology were produced at various penicillin concentrations, up to and including 1,000 U/ml. The induction frequency ranged from 0.02% without supplemental DL-glutamic acid, to 0.17% after its addition. L-colonies were subcultured on fresh, defined medium by the agar block technique and, upon passage, maintained the typical L-form morphology. Inducibility was studied as a function of varying albumin concentration; below 2% albumin, no transformation to the wall-defective variant occurred. More importantly, parental growth was also inhibited in this range. Subsequent studies, using different batches of PVP, have indicated variation in the extent of toxic effects. For example, one particular lot inhibited all parental and L-form growth of strain 474 even in the presence of 2% albumin. Simple dialysis of a 30% solution of PVP reduced the level of toxicity to allow induction. Viable counts indicated that parental survival on PVP L-medium without penicillin was nearly equivalent to that on MH-BAP. In addition, all three strains were found to induce on dialyzed PVP L-medium with frequencies up to 0.3% (Table 3). The gonococcal L-forms of strains 474 and 448 appeared after 2 to 4 days of incubation. The L-form of strain GC#3 was usually slower in appearance; the morphology was less developed when compared to the well-differentiated, fried-egg colonies produced by the other two strains.

The usefulness of sucrose as an osmotic stabilizer, in comparison to PVP, was evaluated with strain 474. Despite the presence of albumin in the medium, parental growth was greatly inhibited. Only 0.24% of the gonococci survived on sucrose L-medium without penicillin in contrast to the recovery on MH-BAP. The sucrose-stabilized L-forms appeared 1 week later than those produced on PVP L-medium. The morphology was characterized by minute granular colonies lacking a less-dense peripheral zone. Strain 474 exhibited a greater than 10-fold increase in conversion to the L-form on PVP L-medium in preference to sucrose L-medium.

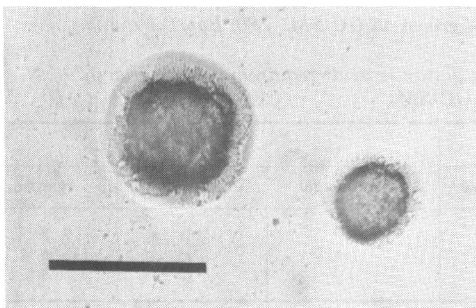

FIG. 4. *L-colony (5 day) of gonococcal strain 474 on PVP L-medium.* ×*80. Bar, 0.1 mm.*

DISCUSSION

Prerequisite to establishing defined cultural conditions for the induction of the L-form of *N. gonorrhoeae* was the adaption of the parental strains to growth in synthetic medium. In 1942, Frantz (6) described a simple medium, composed of salts, glucose, and two amino acids, for the growth of *N. meningitidis*. This laboratory and others (9) have shown this medium to be incapable of supporting the growth of the gonococcus. However, excellent growth was achieved on the addition of 0.4% dialyzed yeast extract to the meningococcal medium, as suggested by Swanson, Kraus, and Gotschlich (12). In this paper, a synthetic medium for *N. gonorrhoeae* is presented in which medium 199 was substituted for yeast extract. Supplemental glutamic acid was eliminated since it was found to inhibit the growth of strain GC#3. A similar phenomenon of strain variation in response to this amino acid was observed by Hunter and McVeigh (8). Possibly, excess glutamic acid may disrupt cell wall synthesis or disturb membrane transport. Although unsupplemented medium 199 was insufficient for growth, two strains achieved equal amounts of growth in media lacking any one of the supplemental components (except the buffer). Further studies are now in progress to determine if the supplements to the tissue culture medium simply provide the osmolarity necessary for growth, since these components do not seem to meet specific growth requirements.

Although by no means a minimal medium, GC-SM supported luxuriant growth of all three strains for over 75 continuous subcultures. The amount of growth as measured by viable count was equal to that observed in Mueller-Hinton broth, surpassing the 15 to 67% yield reported by Hunter and McVeigh (8) for their chemically defined medium. This medium required 96 h of incubation before growth was evident, whereas an 8-h time period was usually sufficient for GC-SM cultures to reach stationary phase. The *Neisseria* chemically defined medium (NCDM) developed by Kenny et al. (9) also incorporated medium 199 but, in contrast to GC-SM, utilized an increased CO_2 tension for broth culture and

TABLE 3. *Growth and induction of the L-form of representative strains of N. gonorrhoeae*

Strain	Parental viable counts (CFU/ml)		L-Form viable count (CFU/ml) L-medium with penicillin[a]	Induction to the L-form (%)
	MH-BAP ($\times 10^8$)	L-medium without penicillin ($\times 10^8$)		
474	2.3	2.7	7.9×10^5	0.3
448	2.8	2.6	9.8×10^4	0.04
GC#3	3.7	3.2	5.8×10^4	0.02

[a] Approximately 10 U of penicillin per ml.

the addition of sodium bicarbonate just prior to inoculation. The latter inconvenience was circumvented in the GC-SM by the use of a second buffer, adjusted to pH 7.0 with NaOH. Most recently Carifo and Catlin (Abst. Annu. Meet. Amer. Soc. Microbiol., 1973, p. 129) have reported a chemically defined medium for *Neisseria* prepared by the combination of 10 complex stock solutions.

Adaption to growth in the synthetic medium resulted in characteristic morphological changes which were first described on complex agar medium by Dienes, Bandur, and Madoff (5). The presence of swollen cocci and numerous large bodies in GC-SM broth indicated the possible spontaneous formation of wall-defective forms, without the addition of inducing agents. Although adapted cultures lost their variant morphology, slight nutritional changes could reinduce large body formation. Similar large body formation in highly complex fluid was noted and observed by Brookes and Hedén (3). The defined cultural conditions set forth in the paper could allow an evaluation of the possible role of an autolytic enzyme system in large body formation and in L-form induction.

The success of growth on solid synthetic medium was correlated with the type of agar employed for solidification. The problem of agar toxicity for the gonococcus has been cited for other defined media. Kenny et al. (10) neutralized the toxicity by the addition of cornstarch, whereas Ionagar no. 2 was used by Hunter and McVeigh (8) in preference to conventional agar. The present data indicated that glutamic acid might be useful for agar neutralization in addition to apparent strain variation in response to different types of agar. The addition of supplemental glutamic acid to purified agar only allowed luxuriant growth of one strain, whereas Ionagar no. 2 supported two out of three gonococcal strains. Since strain GC#3 was inhibited by high glutamic acid concentrations, it was useless for agar detoxification, but the incorporation of 2.0% albumin overcame both problems of agar and glutamic acid toxicity. Agarose proved to be nontoxic for all three strains, and the need for supplemental glutamic acid was eliminated.

Growth on agar was rapid for all three strains; colonies were evident after 18 to 30 h of incubation, compared with 42 to 72 h required on the solid defined medium of Kenny et al. (10). In contrast to previous reports (8, 10, 13), riboflavin and Tween 80 were not inhibitory for growth in the concentrations found in medium 199. Growth studies both in fluid and on solid GC-SM demonstrated that the optimum medium 199 concentration was 0.5×. The concentration of medium 199 used in NCDM was approximately 1.0×, which in GC-SM inhibited the growth of strain GC#3 and significantly decreased the final total viable counts for strains 474 and 448. Perhaps the addition of high concentrations of supplemental salts to GC-SM results in salt toxicity at the 1.0× concentration of medium 199.

The adaption of the gonococcal strains to growth in GC-SM had no effect on their inducibility on the complex PVP induction medium previously described (J. W. Lawson and J. T. Douglas, Can. J. Microbiol., in press). Induction to the L-form was achieved by all three strains on the GC-SM medium plus dialyzed PVP, 2.0% albumin, and penicillin. The principle role of the albumin seemed to be one of further detoxification of both agar and PVP. Both Ionagar no. 2 and DL-glutamic acid were incorporated into the initial induction medium. The positive role of glutamic acid in the neutralization of the deleterious effects of Ionagar no. 2 for strain 474 has already been established both for parental survival and frequency of L-form conversion. Although strain GC#3 was unable to grow in the presence of glutamic acid as long as albumin was present in the medium, parental counts were equal to those on MH-BAP. In studies directed toward the elimination of albumin, agarose has been substituted for Ionagar no. 2 for solidification. At present, no induction to the L-phase has occurred in the absence of albumin.

The osmotic stabilizer, PVP, also proved to be toxic, the extent of which was often dependent on lot number. The average molecular weight of the PVP used was 40,000. A recent study (1) has indicated that in 30K PVP an almost continuous population of molecules, ranging in molecular weight from 1,000 to 200,000, comprises the material. Approximately 12% of the PVP is below13,000, whereas 50% is above 67,000 molecular weight. For lot number 112C-2640 to PVP, which would not support any parental growth even in the presence of albumin, dialysis of an unbuffered PVP solution versus water restored the growth-supportive capacity of the medium to normal levels. To assume, however, that the removal of the PVP size population below 13,000 is sufficient to eliminate all material toxic for L-form induction on the premise that the dialyzed PVP is no longer toxic for the parent coccus, may be incorrect. The L-form, by virtue of its lack of rigid cell wall, may be far more sensitive to the molecular size distribution in PVP. Perhaps only a certain size of PVP molecules is capable of interaction with the osmotically fragile cell in such a way as to prevent lysis. Ashwood-Smith and Warby (1), in their studies on the cryoprotective properties of PVP, found that dialysis greatly enhanced the recovery of *Pseudomonas* F8 after freeze-thaw. They suggested that small-molecular-weight acidic material was responsible for the observed toxicity.

The frequencies of induction routinely obtained on defined PVP L-medium have greatly exceeded that of the complex sucrose L-medium of Roberts (11), but remain significantly lower than those reported by Lawson and Douglas (Can. J. Microbiol., in press) for the complex PVP system. The induction process on defined L-medium has been characterized by rapid patterns of growth and L-form yields sufficient for future studies on the possible role of autolysis as well as antibiotic pressure in the conversion of the L-form. Perhaps the first step in the induction process involves the spontaneous formation of wall-defective variants, subject to favorable environmental conditions, prevented from reversion to the rigid cell-walled parent by penicillin.

A comparison between the use of PVP and sucrose-defined induction media conducted with strain 474 coincided with the previous findings of the complex system. The sucrose induction was denoted by an extended incubation period before growth was evident, small and immature colony morphology, and lower induction frequencies. The defined induction system revealed the toxicity of sucrose for the parental coccus, and presumably its L-form. Employing complex sucrose induction medium, gonococcal L-forms have been isolated from clinical specimens (7). The present data indicated that PVP-stabilized medium would be superior for the isolation of gonococcal wall-defective variants. Other work in this laboratory (in preparation) has shown untreated PVP, and to a lesser extent dialyzed PVP, to be toxic for the induction of the meningococcal L-form in complex medium. The sensitivity of the defined induction system will allow assessment of the nature of the toxicity. The most advantageous clinical isolation medium for osmotically fragile gonococci will require extensively detoxified PVP. Presumably, once toxicity problems have been solved for the defined system, the induction frequencies will hopefully parallel those commonly encountered on the complex PVP medium.

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