

## Characteristics of Atypical *Neisseria gonorrhoeae* from Disseminated and Localized Infections

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Approximately 6% of 1,200 clinical isolates of *Neisseria gonorrhoeae* were atypical because they produced smaller than normal colonies on conventional chocolate agar and fermented glucose weakly. Auxotyping studies indicated that these atypical strains required for growth arginine, uracil, and, in most instances, hypoxanthine. In addition, all of them were susceptible to 0.02 U of penicillin/ml. None of the normal colony isolates, including those susceptible to the same low concentration of penicillin, had the same nutritional characteristics. Atypical strains comprised almost half of the isolates from disseminated infections, but only 5% of those from localized infections. Auxotyping was used to identify the contact of a patient who became reinfected nine times with an atypical gonococcal strain. In addition to its usefulness in such epidemiological studies, this technique has enabled us to distinguish a subgroup of gonococci with apparent increased pathogenicity.

The magnitude of the current gonorrhea epidemic and its widespread geographical distribution have stimulated a search for new methods to distinguish individual strains of *Neisseria gonorrhoeae*. Techniques for typing with antigonococcal sera and testing for susceptibility to bacteriocins (9) have generally been unsuccessful because of their lack of specificity. The method developed by Catlin (2) for auxotyping isolates of *N. gonorrhoeae* on a defined medium has been used by Carifo and Catlin (1) to differentiate clinical isolates of this organism. As a result of their work, a useful tool is now available to study not only nutritional characteristics, but also epidemiological relationships among strains of gonococci.

A defined medium that is somewhat less complex than the one devised by Catlin has been developed by LaScolea and Young (5), primarily as a minimal medium for genetic studies of gonococci. It has also been used by them to separate strains of this organism into a number of phenotypic groups (auxotypes) based upon their requirement for five amino acids included in the complete medium.

Since 1971, we have collected more than 1,200 isolates of *N. gonorrhoeae* from both anogenital and extragenital sites of patients seen at the University of Chicago Hospitals and Clinics. Initially, we were interested in categorizing these strains according to their susceptibility to penicillin and their ability to

produce L-phase variants (M. Bohnhoff and J. A. Morello, Abstr. Annu. Meet. Am. Soc. Microbiol. 1973, G243, p. 66). The published techniques for auxotyping gonococci offered an additional approach to the phenotypic characterization of our strains. Of special interest were 10 gonococcal isolates from a single patient, L.W., that were collected over a 15-month period. These strains were atypical because their colonies were smaller than normal on chocolate agar (CHA), and they produced poor reactions on carbohydrate fermentation medium. Auxotyping has enabled us to categorize such atypical strains more precisely, thereby permitting a comparison of the pathogenesis, epidemiology, and penicillin susceptibilities of typical strains with their atypical counterparts.

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### MATERIALS AND METHODS

**Bacterial strains.** All strains of *N. gonorrhoeae* used in this study were clinical isolates from the Clinical Microbiology Laboratories of the University of Chicago Hospitals and Clinics. The 216 strains examined included isolates from anogenital sites as well as blood, joint fluid, and skin lesions. The organisms were received from the clinical laboratory on CHA plates (Bioquest). All strains were identified as *N. gonorrhoeae* by their appearance on Gram stain, positive cytochrome oxidase reaction, and

acid production from glucose, but not maltose, sucrose, lactose, or fructose, on cystine Trypticase agar base medium (Bioquest). Subcultures were made on CHA and incubated in a candle extinction jar for 18 to 24 h at 37 C. For storage, the entire growth from two CHA plates was suspended into two 1-dram vials containing 1.5 ml of 50% Trypticase soy broth (Bioquest) and 50% heat-inactivated horse serum (Bioquest). The vials were frozen at -84 C. The same vial was used as the source of inoculum for all experiments included in this study. When a subculture showed that the number of viable organisms in the working stock was nearly depleted, it was replenished from growth on CHA, or a new vial was prepared from a subculture on CHA. The second of the original vials remained unopened in the freezer. For most strains, growth on CHA was examined under a stereoscopic microscope ( $\times 10$ ), in order to note whether the colonies appeared typical or atypical, i.e., small and translucent. Weak glucose fermentation reactions were also recorded. Any isolate from blood, joint fluid, or skin was considered to have caused disseminated gonococcal infection (DGI). When an anogenital isolate was received from a patient who had been admitted to the hospital, the chart was reviewed. All in patients who had a positive anogenital culture of *N. gonorrhoeae* as well as signs of arthritis and/or suggestive skin lesions were considered to have DGI even if blood, joint fluid, or skin cultures were negative.

**Auxotyping media.** The basic medium used for auxotyping was the Gonococcal Genetic Medium (GGM) described by LaScolea and Young (5). Ionagar no. 2 (Inolex) and soluble starch (Bioquest) were included. After preparation, 5 ml of the medium was pipetted into plastic petri plates (60 by 15 mm). The plates were stored in plastic bags at 4 C until used, usually within 2 months.

Before auxotyping, strains were screened on a combination of four media consisting of: (i) GGM, which includes isoleucine but not valine; (ii) GGM plus 125  $\mu\text{g}$  of valine/ml (GGMV); (iii) GGM plus 125  $\mu\text{g}$  of valine/ml, minus isoleucine (GGMV-I); and (iv) GGM without isoleucine or valine (GGM-I). For growth of isolates with atypical colony morphology, GGM was supplemented (SGGM) with 11 additional amino acids and myo-inositol (125  $\mu\text{g}/\text{ml}$ ), which are present in the defined medium (NEDA) described by Catlin (2). The amino acids were alanine, asparagine, glutamine, glycine, histidine, leucine, lysine, phenylalanine, threonine, tryptophan, and valine (Calbiochem). Stereoisomers were of the L configuration.

**Auxotyping procedure.** Gonococci grown overnight on CHA were suspended in phosphate-buffered saline, pH 7.2, to a concentration of approximately  $10^8$  colony-forming units/ml. A 0.001-ml amount of this suspension was then streaked with a calibrated inoculating loop onto one-fifth of each plate of the appropriate auxotyping medium. Auxotypes were determined on GGM by the single omission from individual plates of arginine, aspartic acid, glutamic acid, isoleucine, methionine, proline, and serine. Those strains that required arginine on

GGM were tested for their requirement for hypoxanthine and uracil. On SGGM, auxotypes were determined by the separate omission of arginine, histidine, isoleucine, leucine, lysine, methionine, proline, serine, valine, hypoxanthine, thiamine pyrophosphate, and uracil. All atypical strains and normal DGI strains that required arginine were also examined on SGGM in which ornithine was substituted for arginine. The plates were incubated at 37 C for a total of 3 days in candle extinction jars and examined daily with a stereoscopic microscope ( $\times 10$ ) using transmitted illumination. The numbers of colonies were quantitated roughly from 0 to 4+, but only the presence or absence of any growth after 48-h incubation was used for compiling the results. Even when only a few colonies were seen at the site of inoculation, they were considered to represent growth of the organism when it was apparent that no contamination or cross-feeding from adjoining organisms on the plate had occurred.

**Penicillin susceptibility studies.** The medium for penicillin susceptibility studies was Trypticase soy agar (Bioquest) supplemented with 7.5% horse serum. All gonococcal isolates were screened initially on plates containing 0.02, 0.1, and 0.6 U of benzyl penicillin/ml. Growth from a CHA plate incubated for 18 to 24 h was suspended in Trypticase soy broth with 7.5% horse serum and diluted to a concentration of approximately  $10^7$  colony-forming units/ml. A 0.01-ml aliquot of this suspension was streaked with a calibrated loop onto one-fifth of each of the penicillin-containing agar plates. The plates were incubated in candle extinction jars at 37 C, and the amount of growth present after 48 h was recorded. More precise determinations of the minimal inhibitory concentration were made on those strains selected for auxotyping studies. The minimal inhibitory concentration was the concentration of penicillin at which fewer than 100 colonies grew within 48 h.

## RESULTS

**Relationship of growth on GGM to colony morphology.** Initial auxotyping determinations were made on GGM from which individual amino acids were omitted. However, a number of strains, including those isolated from patient L. W., failed to grow on this medium even when all of the prescribed amino acids were included. It was observed that almost all isolates which did not grow on GGM produced small, translucent, slowly growing colonies on CHA that were often difficult to speciate biochemically because of their poor growth and weak acid production from glucose. A review of our culture collection indicated that 90 strains had been noted to produce small colonies and/or weak fermentation reactions. Eighty-seven of these were unable to grow on GGM and were examined in greater detail.

**Effect of addition of valine to GGM.** Al-

though isoleucine is present in GGM, valine is not included. The interrelationship of these two branched-chain amino acids on the growth of the small colony type of gonococci was examined on GGMV, GGMV-I, and GGM-I. Of the 87 small-colony gonococcal isolates that would not grow on GGM, i.e., in the presence of isoleucine but absence of valine, four were able to grow in the presence of valine but absence of isoleucine (GGMV-I). When both isoleucine and valine were included in the medium (GGMV), 56 of the 87 strains grew to some extent, and 41 of these 56 also grew when both of the amino acids were omitted (GGM-I). Growth of the gonococci was always better in the presence of isoleucine and valine than in their absence.

**Effect of additional supplementation of GGM.** Of the 31 remaining strains of gonococci that did not grow on GGMV, 29, including all 10 isolates from L.W., grew on SGGM. The other two strains did not grow on any of the experimental media. In most instances growth on SGGM was quite luxuriant, and it was sometimes better than growth on CHA. The 56 strains that grew on GGMV also grew well on SGGM.

**Auxotypes of *N. gonorrhoeae* that did not grow on GGM.** The 85 strains of gonococci that grew on SGGM but not GGM were auxotyped on SGGM from which amino acids, uracil, hypoxanthine, or thiamine pyrophosphate were individually omitted. The results are summarized in Table 1. It is striking to note that 82 of the 85 strains required arginine, alone or in combination with other amino acids, and all

except eight strains required either uracil or hypoxanthine and uracil. None of the strains were found to require thiamine pyrophosphate, and all except one strain ( $\text{Arg}^- \text{Ile}^- \text{Ura}^-$ ) grew when ornithine was substituted for arginine. The 10 isolates from L.W. and one strain isolated from her sexual contact required arginine, leucine, hypoxanthine, and uracil. Eight strains [one from L.W., one from L.W.'s contact, the five  $\text{Arg}^- (\text{Orn}^-) \text{His}^-$  strains, and the  $\text{Lys}^-$  strain] were auxotyped on NEDA by B. W. Catlin. In each instance, the requirements found with SGGM were confirmed (B. W. Catlin, personal communication). The auxotypes of strains isolated from patients with DGI are indicated separately. There appears to be no uniformity in their amino acid requirement.

**Auxotypes of *N. gonorrhoeae* that grew on GGM.** All 131 gonococcal strains that grew on GGM were auxotyped on GGM and/or SGGM. Six of the 22 strains tested on both media appeared to have a requirement for isoleucine when this amino acid was omitted from GGM. However, when the same strains were tested on SGGM with isoleucine omitted, growth was always confluent, and therefore this amino acid was not considered to be a requirement for them. The remaining nutritional requirements of these strains were identical regardless of whether auxotyping was performed with GGM or SGGM. The results are compiled in Table 2. In marked contrast to the group of isolates that did not grow on GGM, a majority of these strains did not require arginine. However, three of the strains which did require

TABLE 1. Nutritional requirements of *N. gonorrhoeae* that did not grow on GGM

Requirement		No. of isolates			Percentage of total
Amino acids	Nitrogen bases	Anogenital	DGI	Total	
Arg(Orn) <sup>a</sup>	Hyx-Ura	24	5	29	34.1
Arg(Orn)-Leu	Hyx-Ura	14	3	17 <sup>b</sup>	20.0
Arg(Orn)-Pro	Hyx-Ura	5	1	6	7.1
Arg(Orn)-Ile	Hyx-Ura	4	1	5	5.8
Arg(Orn)-Ser	Hyx-Ura	4	1	5	5.8
Arg(Orn)-Ile-Val	Hyx-Ura	1	0	1	1.2
Arg(Orn)-Leu-Val	Hyx-Ura	0	1	1	1.2
Arg(Orn)-Ile-Ser-Val	Hyx-Ura	2	0	2	2.4
Arg(Orn)-Ile-Leu-Ser	Hyx-Ura	0	1	1	1.2
Arg(Orn)	Ura	8	1	9	10.6
Arg-Ile	Ura	1	0	1	1.2
Arg(Orn)-His		5	0	5	5.8
Pro-Leu		2	0	2	2.4
Lys		1	0	1	1.2

<sup>a</sup> Ornithine can replace the requirement for arginine.

<sup>b</sup> Includes 10 isolates of patient L.W.

TABLE 2. Nutritional requirements of *N. gonorrhoeae* that grew on GGM

Requirement		No. of isolates			Percentage of total
Amino acids	Nitrogen bases	Anogenital	DGI	Total	
None		35	12	47	35.9
Pro		42	6	48	36.6
Arg <sup>a</sup>		21	2 <sup>b</sup>	23	17.6
Arg <sup>a</sup> -Pro		8	0	8	6.1
Arg <sup>a</sup> -Ser		2	0	2	1.5
Arg	Hyx-Ura	1	2	3	2.3

<sup>a</sup> Requirement for ornithine not examined for every strain.

<sup>b</sup> Ornithine replaced the requirement for arginine.

arginine also required hypoxanthine and uracil and could not utilize ornithine in place of arginine.

**Relationship of auxotype and colony morphology of *N. gonorrhoeae* isolated from localized and disseminated infections.** Initially, it appeared that strains of gonococci could be divided into two groups depending upon their ability or inability to grow on GGM. Most of those strains that do not grow on this medium (GGM-negative) also produce smaller and more translucent colonies on CHA. An examination of the auxotypes of the 85 GGM-negative strains (Table 1) reveals that all but eight require either arginine and uracil (AU), or arginine, hypoxanthine, and uracil (AHU). However, the eight exceptions were found to require compounds not present in GGM (histidine, leucine, or lysine). In experiments not shown here, the separate addition of histidine, leucine, or lysine to GGM allowed the strains that require these amino acids to grow on this medium. In contrast, even though the AHU combination is present in GGM, the addition of leucine to this medium did not allow growth of strains that require leucine and AHU. Auxotyping on SGGM had shown that, of the strains that did not grow when valine was added to GGM, all but two required compounds not present in GGM. It is possible, therefore, that addition of valine as well as leucine might have permitted growth of these strains. It appears that the eight strains that do not have a requirement for uracil or hypoxanthine and uracil (Table 1) are more closely related to the GGM-positive than the GGM-negative strains, and we consider these strains to belong to the former group. Of the remaining GGM-positive strains (Table 2), three behave more like the GGM-negative isolates, for they require AHU and also produce small colonies on CHA.

On the basis of nutritional requirements and colonial appearance, therefore, we have assigned the GGM-positive and negative strains

to either of two groups that we consider "atypical" or "normal." The atypical strains are those that require arginine or arginine plus other amino acids, and uracil or uracil plus hypoxanthine (AHU/AU). All of these strains also produce small colonies on CHA. Seventy-one of the strains studied have these characteristics if the L.W. isolates are considered as one strain.

Table 3 indicates the type of infection from which the gonococcal strains were isolated. Of a total of 1,216 isolates, 1,180 were from anogenital infections and 36 were from DGI. Among the anogenital isolates, 55 or 4.7% were atypical strains, but among the isolates from DGI, 16 or 44.4% were atypical.

**Penicillin susceptibilities of *N. gonorrhoeae* strains.** The penicillin susceptibilities of the strains of gonococci isolated from DGI are illustrated in Fig. 1. All of the atypical isolates, but only half of the normal strains, were susceptible to 0.02 U of penicillin/ml. Atypical gonococci isolated from anogenital infections were found to be universally susceptible to this same low concentration of penicillin. Among our entire collection of 1,180 normal anogenital isolates, approximately 13% were susceptible to 0.02 U of penicillin/ml (data not shown). For reasons described in the next section, approximately one-third of the normal genital isolates chosen for auxotyping were selected because screening showed them to be as susceptible to penicillin as the atypical strains. The remainder were consecutive isolates.

TABLE 3. Occurrence of atypical isolates of *N. gonorrhoeae*

Type of infection	Total no. of isolates	Atypical no. of isolates	Atypical/total (%)
Anogenital	1,180	55	4.7
DGI	36	16	44.4

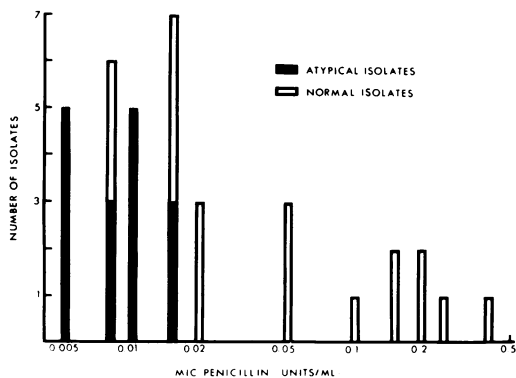


FIG. 1. Penicillin susceptibilities of *N. gonorrhoeae* isolates from disseminated infections.

**Relationship of penicillin susceptibility to auxotype of normal gonococcal isolates.** Because all of the atypical strains of gonococci require arginine and are susceptible to 0.02 U of penicillin/ml, normal strains were selected according to their susceptibility or relative resistance to this antibiotic and were examined for a correlation between the arginine requirement and susceptibility to penicillin. In Fig. 2, it can be seen that among normal isolates, penicillin susceptibility is unrelated to strain auxotype. In contrast to the atypical strains, only 14 of the 68 normal susceptible strains required arginine.

## DISCUSSION

The auxotyping technique for characterization of *N. gonorrhoeae* promises to become a method with broad applications. Although important epidemiological information can be obtained by this means, it is evident that care must be taken to obtain results that are reproducible and comparable with those of other workers. Our initial studies were performed with GGM because it was less complex than the medium devised by Catlin. Providing that their nutritional requirements were present, GGM supported the growth of normal colony strains of gonococci, but not of strains that produced small, atypical colonies on CHA. Many of the atypical isolates grew only after valine was added or isoleucine was omitted from GGM. Thus, in *N. gonorrhoeae*, the biosynthetic pathways for isoleucine and valine may be interdependent. In *Escherichia coli*, the interrelationship of these pathways and their regulation have been well documented (6, 7).

In studies performed on GGM, LaScolea and Young noted that the isoleucine genetic marker was unstable in some strains (5). Carifo and Catlin found that they could not obtain con-

sistent results when this amino acid was omitted from auxotyping media, and it was not included as one of their markers (1). We also experienced some difficulty interpreting results when valine and serine as well as isoleucine were individually omitted from the medium. These three amino acids were considered requirements for our strains only after the organisms failed to grow on two or more lots of media from which isoleucine, valine, or serine was omitted. In some instances, older strains appeared to lose their requirements for isoleucine, valine, and serine after repeated replenishment of the stock culture vial from growth on CHA. We plan to study the unopened frozen sample of our atypical strains in an effort to clarify the effect of repeated subculture on the stability of their auxotypes. Until further work is done in this area, it seems reasonable to exclude determination of the requirement for isoleucine, valine, and serine from routine auxotyping.

Because our studies were not performed with single-colony isolates, the possibility exists that inconsistencies in growth on auxotyping medium might represent the presence of a second gonococcal strain from a mixed infection. We encountered such inconsistencies only when testing the requirement for isoleucine, valine, and serine. The results with all other amino acids were unambiguous. In experiments not described here, we have been able to detect two gonococcal strains from a single specimen by their different colonial morphologies and penicillin susceptibilities. To our knowledge, none of the strains in the study reported here represents mixed cultures.

Although not all of our normal colony strains of *N. gonorrhoeae* were consecutive isolates, the distribution of their auxotypes appears to be similar to that reported by Carifo and Catlin for Milwaukee strains, and by LaScolea and Young for Rochester strains. The two major auxotypes consist of gonococci that require none of the

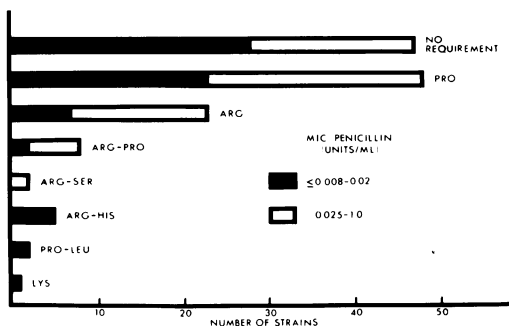


FIG. 2. Relationship of penicillin susceptibility to auxotype of normal gonococcal isolates.

amino acids tested or only proline. A requirement for arginine alone constitutes the third largest group.

The strains that produce small colonies on CHA appear to be quite distinct from those that produce normal colonies. Although it occurred to us that the small colonies might represent simply those described by Kellogg as type 1 or 2 (T1-2) (3), after repeated, nonselective subculture, they dissociated into larger, but still relatively small colonies that morphologically resembled Kellogg's types 3 or 4 (T3-4) colonies (Fig. 3). The existence of T1-2 and T3-4 colonies among these strains was confirmed by adsorption of red cells (8) to the former, but not to the latter types (data not shown). The atypical colony strains are distinguished also by their extreme susceptibility to penicillin and their requirement for AU, and in most instances, hypoxanthine. None of the normal colony strains had these nutritional requirements even though the sample tested was biased by choosing isolates that were susceptible to the same low concentration of penicillin.

Recently, Knapp and Holmes (4) reported on the incidence of *N. gonorrhoeae* with unique nutritional requirements in patients with DGI. These strains were isolated from 89% of disseminated infections, as well as 39% of uncomplicated infections. All were susceptible to 0.015  $\mu\text{g}$  or less of penicillin/ml, and required the presence of AHU for growth. Although the colony morphology of their isolates is not described, it is likely that they correspond to our

atypical AHU/AU strains. However, their population of gonococci apparently differs from ours since we isolated atypical gonococci from 44% of patients with DGI, but only 4.7% of patients with uncomplicated infections. Despite this difference, it is clear that the strains that require AHU/AU produce disseminated infections much more frequently than would be predicted by their incidence in the population. The possible relationship between the metabolic deficiencies of these strains and their increased invasiveness is not clear at this time. We are planning to study the infections produced by atypical and normal strains of gonococci in chicken embryos inoculated by different routes in an attempt to clarify the factors related to pathogenicity of the atypical strains.

Knapp and Holmes noted that only their strains that require AHU were susceptible to 0.015  $\mu\text{g}$  or less of penicillin/ml. Strains susceptible to higher concentrations of antibiotic had other nutritional requirements. In our studies, all of the AHU/AU (atypical) strains were susceptible to comparable concentrations of penicillin (0.02 U/ml). In addition, among the normal colony isolates, there were penicillin-susceptible strains in all other auxotype groups except  $\text{Arg}^- \text{Ser}^-$  which contained only two isolates (Fig. 2). Unlike Knapp and Holmes, therefore, we did not find an absolute correlation between penicillin susceptibility and the AHU requirement.

A major problem encountered by Knapp and Holmes during their auxotyping studies was

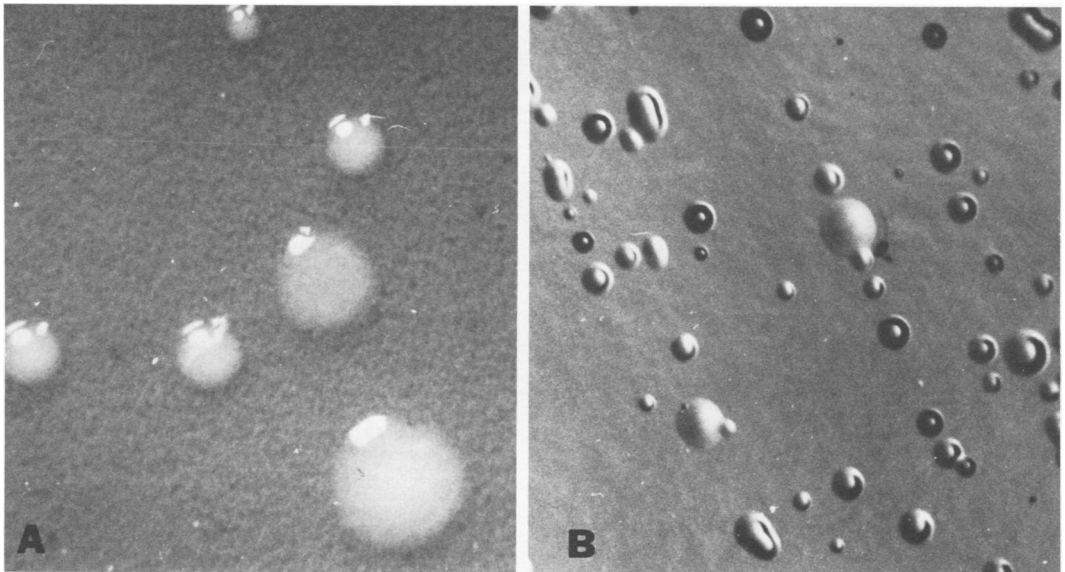


FIG. 3. Representative colonies of normal (A) and atypical (B) strains of *N. gonorrhoeae* grown on CHA ( $\times 15$ ). T1-2 and T3-4 colony types can be seen for each strain.

the toxicity of ethanol-washed purified agar for their atypical strains. Auxotyping was possible only on methanol-washed purified agar supplied to them by Catlin. With our medium, which contained unwashed Ionagar no. 2 and soluble starch (5), no such toxicity problem was encountered. Presumably, the starch is present to neutralize toxic components in the medium (5), but the possibility must be considered that it might add undefined nutrients to the medium resulting in inaccurate auxotype determinations. It is mandatory, therefore, to prescribe quality control for each new lot of medium with a set of reference strains. Those strains that were used by Catlin are obtainable through the American Type Culture Collection and the National Collection of Type Cultures (1). Presently, the commercially available supply of Ionagar is almost depleted and comparable agar may not be produced again. We are now examining a number of agar preparations with the hope of finding a suitable alternative to Ionagar that does not require extensive purification.

The potential clinical usefulness of auxotyping is illustrated by our experience with the 10 urogenital isolates from L.W., a 24-year-old diabetic female. This patient's initial episode was DGI as determined by her clinical history and presentation. Presumably, she was successfully treated since follow-up cervical cultures during her hospital stay yielded no gonococci. Her subsequent episodes of cervical infection were asymptomatic and detected only by isolation of *N. gonorrhoeae* from cultures obtained at the time of her routine visits for management of her diabetes. All 10 of her isolates required AHU and leucine for growth on SGGM. Careful history-taking elicited the name of a sexual contact whose urethral culture grew a proline-requiring strain of *N. gonorrhoeae* with normal colony morphology. Upon repeated questioning, she named a second contact whose urethral culture yielded gonococci with the Arg<sup>-</sup>(Orn<sup>-</sup>)Leu<sup>-</sup>Hyx<sup>-</sup>Ura<sup>-</sup> requirement. Treatment of this contact has resulted, to date, in "permanent" cure for L.W. In this instance, auxotyping enabled us to identify the correct source of the L.W. strain of gonococcus and to determine that her carriage of this strain represented reinfection rather than relapse due to inadequate therapy or perhaps to factors related to her diabetic condition. The leucine marker was helpful in distinguishing this strain from the majority of atypical isolates that require only AHU. Obviously, if additional reproducible markers can be used in the auxotyping technique, greater

discrimination among strains of gonococci will be possible.

When properly performed and interpreted, auxotyping provides valuable information concerning metabolism and epidemiological spread of *N. gonorrhoeae*. In our hands, it has indicated that a group of organisms with atypical colonial characteristics and apparent increased pathogenicity share common nutritional requirements. In addition, auxotyping enabled us to establish that a patient from whom we repeatedly isolated the same strain of gonococcus had been reinfected each time by a single contact who was an asymptomatic carrier of this strain. This technique should continue to play an important role in the expanding research on *N. gonorrhoeae*.

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