Brit. J. vener. Dis. (1975) **51**, 382

Cultivation of Type 1 *N. gonorrhoeae* in liquid media

K. CHAN, G. M. WISEMAN, AND J. D. CAIRD
Department of Medical Microbiology, University of Manitoba, Winnipeg R3E OW3, Canada

In the light of current interest in *Neisseria gonorrhoeae*, attempts have recently been made to develop a growth medium which would permit production of the organisms in bulk. In these studies it was important to ensure that stability of colonial types was maintained during growth. Kellogg, Peacock, Deacon, Brown, and Pirkle (1963) observed that the gonococcus underwent morphological variation in which four colonial types were observed. These are Types 1 and 2, virulent for man and for fertile hens' eggs, and relatively avirulent Types 3 and 4. Since that time, Jephcott and Reyn (1971) have detected another colonial type, which they have designated Type 5, which Chan and Wiseman (1975) have shown to be avirulent in eggs. Inoculation of liquid media with avirulent gonococci has generally resulted in the recovery of identical organisms after incubation, but inoculation with virulent Type 1 and 2 cells produces a large proportion of Type 3 and 4 cells at the end of growth. Kenny, Ashton, Diena, and Greenberg (1967) devised a chemically defined medium (NCDM) which they said would support growth of Type 1 gonococci. Jephcott (1972) could not confirm their observations and found that NCDM medium was the least successful of several liquid media in maintaining Type 1 colonial stability. Jephcott also showed that the biphasic (BP) medium of Gerhardt and Heden (1960), which consisted of dextrose starch agar overlaid with dextrose starch peptone solution supported growth but was unable to maintain stability of a Type 1 inoculum at a CO_2 tension of 10 per cent.

The present study compares the growth and stability of gonococcus types in several liquid media at various concentrations of CO_2.

Material and methods

Maintenance and preservation of strains

Two strains of *N. gonorrhoeae*, F62 and 3956, were investigated. The F62 strain was obtained from Dr. D. S. Kellogg of the Communicable Diseases Centre in Atlanta, USA, and 3956 was isolated from a patient at the Manitoba Provincial Laboratories in Winnipeg.

Supported by a National Health Grant

The strains were grown on GCBD medium (Kellogg and others, 1963), which is composed of Difco GC medium base supplemented with dextrose (2 per cent.), L-glutamine, and thiamine pyrophosphate. Inoculated plates were incubated for 20 hrs at 37°C in a Hotpak CO_2 incubator under 5 per cent. CO_2 tension and 95 per cent. relative humidity. Colonial types were identified with a Bausch and Lomb Model 7 Stereomicroscope and selectively subcultured daily on to fresh GCBD medium. The microscope was equipped with a substage source of diffuse light. This arrangement was coupled with oblique overhead illumination by means of a Bausch and Lomb Illuminator (cat. no. 31-35-47). Differentiation of colonies under these lighting conditions was made according to criteria set out by the Kellogg group and by Jephcott and Reyn (1971). Type 1′ newly described by Chan and Wiseman (1975), was also investigated. Type 1′ resembles Type 1 and 2 colonies in physical characteristics but the colonies are granular, slightly crenated, and deeper gold in colour under oblique lighting. The new type also possesses pili and is virulent in eggs.

Isolated strains, in addition to daily subculturing, were preserved in ampoules submerged in liquid nitrogen according to the method of Ward and Watt (1971). Strains to be frozen were suspended in a solution of 8 per. cent. (v/v) glycerol and 1·0 per cent. (w/v) proteose peptone in water.

Measurement of CO_2 tension

The CO_2 concentration in the chamber of the shaker was measured with the 'Kwik-Chek' apparatus (Burrell Corp., Pittsburgh, PA, USA).

Viable counts

Numbers of viable bacteria were expressed as Colony Forming Units (CFU) and were determined according to the method of Miles, Misra, and Irwin (1938). The proportion of gonococcus types in the suspensions was also observed in most cases.

Cultivation of gonococci in liquid media

Three liquid growth media were used in this study: the BP medium of Gerhardt and Heden (1960), NCDM of Kenny and others (1967), and Enriched Single Phase (ESP) medium devised in our laboratory. The last consisted of Difco GC medium base devoid of agar and starch to which were added Lankford supplement (as given by Kellogg and others, 1963) at a concentration of

5 per cent. (v/v) and 1 per cent. (v/v) 'Isovitalex' (Baltimore Biological Laboratories, Baltimore, MD, USA). The BP medium was composed of 100 ml. Difco dextrose starch agar base overlaid with 25 ml. dextrose starch peptone solution in a 250 ml. flask.

After 20 hrs growth on GCBD plates, colonial types were harvested in sterile saline. Flasks of NCDM, BP, and ESP medium (25 ml. liquid medium per 250 ml. flask) were incubated overnight at the CO_2 tension to be investigated and inoculated with 1 ml. of a suspension of gonococci so that the final concentration of cells was about 10^6 CFU/ml. The flasks were placed in a New Brunswick reciprocating shaker-incubator (Model R-25) and shaken at 160 r.p.m. for 30 hrs at 37°C. Compressed air and the appropriate amount of CO_2 were pre-mixed and fed to the incubator chamber, and the concentration of CO_2 above the flasks was measured periodically. No attempt was made to assay CO_2 content in the media. Viable counts were performed at fixed intervals as given under Results.

In one experiment, flasks which contained 100 ml. NCDM were inoculated with a suspension of cells (final concentration about 10^5 CFU/ml.), following the methods of Kenny and others (1967) as closely as possible. The flasks were gassed for 30 sec. with sterile 10 per cent. CO_2 in air and sealed with rubber stoppers. The inoculated medium was incubated in the shaker at 150 r.p.m. for 30 hrs at 37°C.

Results

Flasks of BP and ESP media were incubated simultaneously in CO_2 concentrations of 0, 6, 12, and 16 per cent., and were then removed from the shaker at 4-hrly intervals up to 30 hrs in some experiments, so that viable counts could be made. The CO_2 tension was re-established immediately after the removal of a sample.

The results are shown in Figs 1 to 7.

In Figs 1A and 1B, no multiplication of 3956 or F62 gonococci occurred and no living cells of 3956 remained after 24 hrs incubation in air. At the end

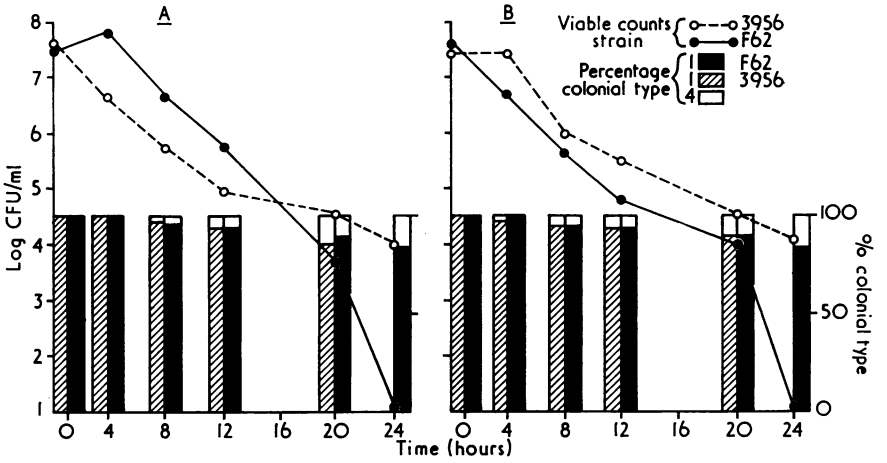

FIG. 1 *Cultivation of type* 1 *gonococci in liquid media in air. ESP medium* (A), *BP medium* (B)

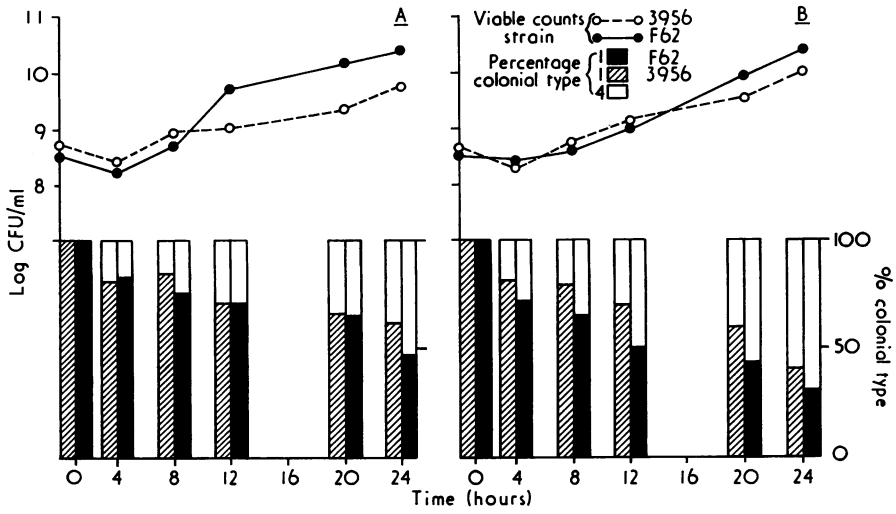

FIG. 2 *Cultivation of type* 1 *gonococci in liquid media in* 6 *per cent.* CO_2. *ESP medium* (A), *BP medium* (B)

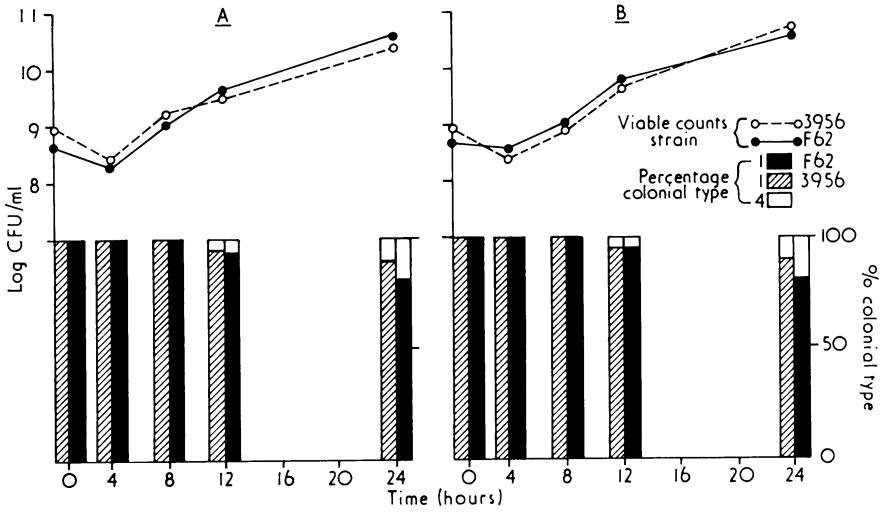

FIG. 3 *Cultivation of type 1 gonococci in liquid media in 12 per cent. CO_2. ESP medium* (A), *BP medium* (B)

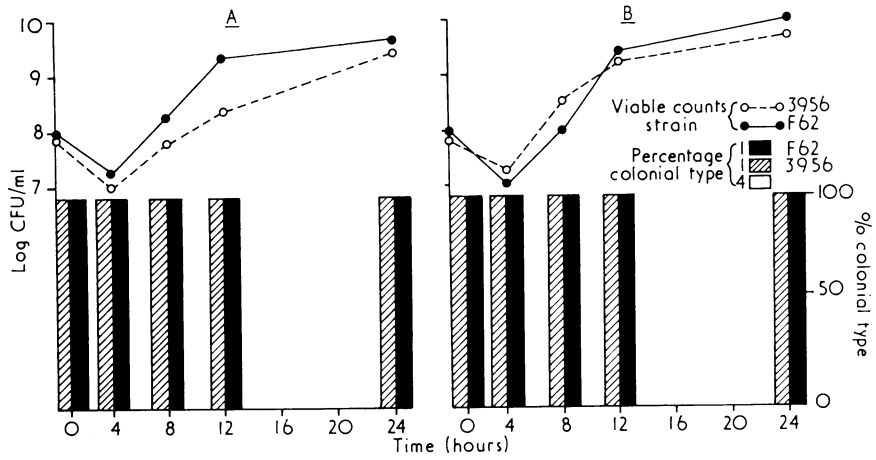

FIG. 4 *Cultivation of type 1 gonococci in liquid media in 16 per cent. CO_2. ESP medium* (A), *BP medium* (B)

of 20 hrs, about 15 per cent. of the survivors were Type 4.

In Figs 2A and 2B (6 per cent. CO_2), some multiplication of both strains of Type 1 cells was observed at 24 hrs but 40 to 70 per cent. of the organisms were Type 4.

At a 12 per cent. concentration of CO_2 (Figs 3A and 3B), both media support medultiplication of the two strains to the same extent as that observed with 6 per cent. CO_2 levels. Colonial stability of Type 1 was improved, about 10–20 per cent. Type 4 being identified.

Carbon dioxide concentrations of 16 per cent. (Figs 4A and 4B) also supported growth to an extent not greatly different from that attained at lower CO_2 tensions, but Type 1 cells of both F62 and 3596 were stable. No avirulent colony types were detected at the end of 24 hrs.

There would seem to be little difference in the results obtained in ESP and BP media, except that the latter did not support growth of smaller inocula as shown in Figs 5A and 5B. Generally, increased CO_2 concentrations caused a decrease in the generation time (Table I).

TABLE I *Generation time of gonococci in liquid media incubated at different CO_2 tensions*

		Generation time (hrs) at per cent. CO_2 concentration of:		
Strain	*Medium*	6	12	16
F62 (Type 1)	ESP	4·0	1·3	1·0
	BP	4·0	1·4	1·3
3956 (Type 1)	ESP	4·0	2·2	1·7
	BP	2·4	2·4	0·9
3956 (Type 1', 2, 4)	ESP	—[a]	—	1·3
3956 (Type 4)[b]	NCDM	—	—	1·5

[a]Not done [b]The only stable type

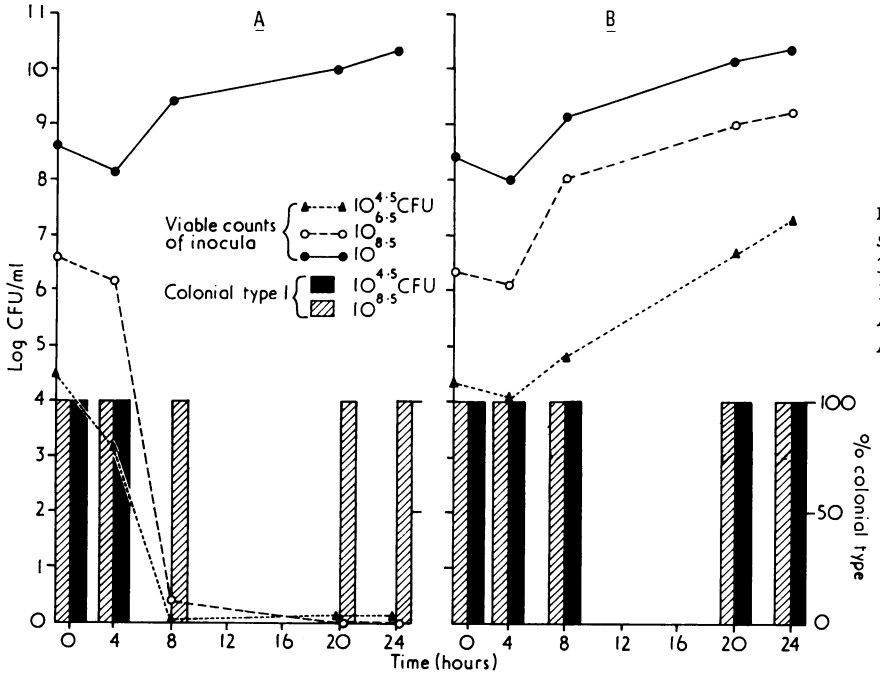

FIG. 5 *Effect of inoculum size on cultivation of strain* 3956 *type* 1 *gonococci in* 16 *per cent.* CO_2. *BP medium* (A), *ESP medium* (B)

Under 16 per cent. CO_2 tension, NCDM was equally successful in permitting growth of gonococci, but failed to maintain stability of a Type 1 inoculum, since 60 per cent. Type 4 cells were found after 24 hrs (Fig. 6).

Other colonial variants investigated were Types 1′, 2, and 4. In ESP medium under 16 per cent. CO_2, all types were stable with growth patterns resembling those of Type 1 under the same conditions (Fig. 7).

Conditions of inoculation and incubation recommended by Kenny and others (1967) were adhered to in a final experiment in which growth and colonial stability in the three media were investigated. As shown in Table II, 30-sec. gassing with a CO_2 concentration of 10 per cent. in air was inadequate for maintenance of Type 1 cells of these strains of gonococci. Growth and stability of Type 4 cells from a Type 4 inoculum, however, matched that obtained under conditions of continuous gassing.

TABLE II *Effect of* 30-*sec. gassing with* 10 *per cent.* CO_2 *on growth of the gonococcus in liquid media**

		Initial Type 1 *inoculation*		*Initial Type* 4 *inoculation*	
Medium	*Time (hrs)*	*Type* 1	*Type* 4	*Type* 1	*Type* 4
NCDM	0	10^5†	0	—‡	—
	30	0	$5{\cdot}7\times10^6$	—	—
ESP	0	$6{\cdot}6\times10^4$	0	0	$7{\cdot}6\times10^{10}$
	30	$7{\cdot}6\times10^5$	$3{\cdot}2\times10^5$	0	$3{\cdot}3\times10^6$
BP	0	$6{\cdot}6\times10^4$	0	0	$7{\cdot}6\times10^6$
	30	4×10^6	$4{\cdot}8\times10^6$	0	$1{\cdot}2\times10^6$

*Conditions of incubation and inoculation described by Kenny and others (1967) were followed
†Viable counts (CFU/ml.)
‡Not done

Discussion

Our observations show that CO_2 tension is important in the maintenance of gonococcus Type 1 colonial stability. Jephcott (1972) also pointed to the critical nature of CO_2 concentration, but found that the growth of Type 1 cells in liquid media under his experimental conditions still favoured the appearance of a large proportion of altered colonial types.

We found no differences between BP and ESP media in terms of yield, and both maintained Type 1 organisms through 30 hrs growth provided that the CO_2 concentration above the flasks was kept at 16 per cent. No other variants were detected during this period. Lower CO_2 tensions of the order of 6 to 12 per cent. were ineffective in promoting Type 1 stability, although Type 4 cells were less dependent upon CO_2 levels.

The basis of discrepancies in results obtained ourselves, by Jephcott, and by Kenny and others (1967) is possibly due to the use of strains of gonococci with differing growth requirements. Catlin (1973) and Carifo and Catlin (1973) have shown that gonococci may be separated into groups

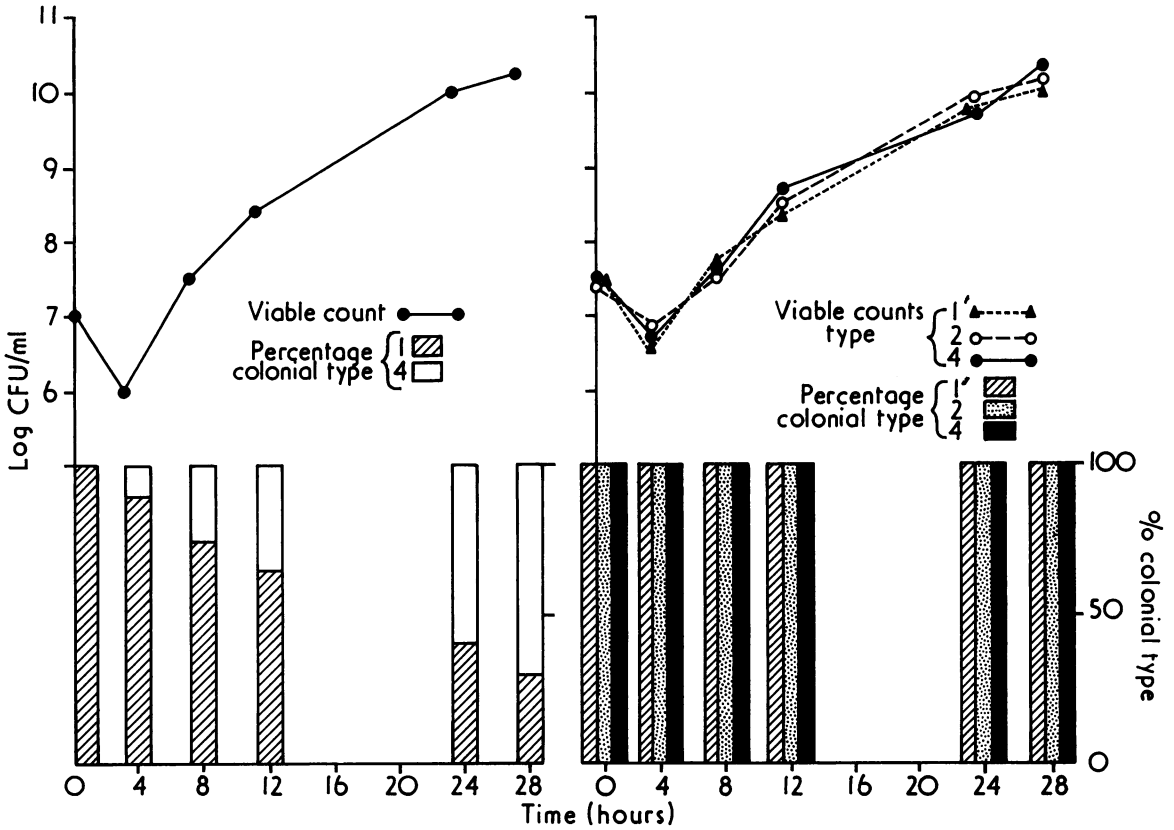

FIG. 6 *Cultivation of strain* 3956 *type* 1 *gonococci in NCDM medium at* 16 *per cent.* CO_2

FIG. 7 *Cultivation of strain* 3956 *gonococci in ESP medium at* 16 *per cent.* CO_2

of varying nutritional patterns in respect to nucleotides and amino acids. This may well apply to CO_2 requirements also.

Summary

The effect of CO_2 concentration on the growth and colonial stability of Type 1 *Neisseria gonorrhoeae* has been investigated. Carbon dioxide at a concentration of 16 per cent. in air above flasks incubated in a shaker was effective in supporting growth and 100 per cent. colonial stability of Types 1, 1', 2, and 4. Lower CO_2 tensions increased the generation time of the strains and were less effective in maintaining the stability of virulent variants. Of several liquid media tested, Enriched Single Phase medium, which consists of Difco GC medium base (devoid of agar and starch) to which Lankford supplement and 'Isovitalex' have been added, was the most suitable for use with small inocula.

References

CARIFO, K., and CATLIN, B. W. (1973) *Appl. Microbiol.*, **26,** 223

CATLIN, B. W. (1973) *J. infect. Dis.*, **128,** 178

CHAN, K., and WISEMAN, G. M. (1975) *Brit. J. vener. Dis.*, **51,** 251

GERHARDT, P., and HEDEN, C. G. (1960) *Proc. Soc. exp. Biol. (N.Y.)*, **105,** 49

JEPHCOTT, A. E. (1972) *Brit. J. vener. Dis.*, **48,** 369

—— and REYN, A. (1971) *Acta path. microbiol. scand.*, **79B,** 609

KELLOGG, D. S., PEACOCK, W. L., DEACON, W. E., BROWN, L., and PIRKLE, C. I. (1963) *J. Bact.*, **85,** 1274

KENNY, C. P., ASHTON, F. E., DIENA, B. B., and GREENBERG, L. (1967) *Bull. Wld Hlth Org.*, **37,** 569

MILES, A. A., MISRA, S. S., and IRWIN, J. O. (1938) *J. Hyg. (Lond.)*, **38,** 732

WARD, M. E., and WATT, P. J. (1971) *J. clin. Path.*, **24,** 122