SHORT COMMUNICATIONS

The Effect of Atmospheric Conditions on the Growth of *Haemophilus gallinarum* in a Defined Medium

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INTRODUCTION

The nutritive requirements of several species of the genus *Haemophilus* have been studied in great detail. In many cases blood products may be completely replaced by yeast extract or pure compounds.

Haemophilus gallinarum is a fastidious organism which is usually grown using complex media containing serum or other fresh blood products. It grows on tryptose agar without serum or feeder cultures provided that NADH is incorporated into the medium (Page, 1962). However, most investigations report that growth as a pure culture in broth media requires the presence of serum.

Delaplane & Stuart (1939) were able to grow *H. gallinarum* in broth without blood products if the organism was grown as a symbiont to live yeast. Slight growth was obtained aerobically in peptone water by Gregory (1944) when filtered yeast extract was added to the broth. There appears to be no requirement for increased CO₂ tension when the organism is grown in broth containing chicken serum, but growth on agar plates containing chicken serum requires CO₂ (Rimler, unpublished).

This study was initiated to determine whether incubation of broth cultures under increased CO₂ tension would substitute for the growth-supporting effect provided by serum, and whether *H. gallinarum* would grow under increased CO₂ tension in a chemically-defined medium similar to those described for other members of the genus *Haemophilus*.

METHODS

Media. Maintenance medium (MM) used for the maintenance of cultures and preparation of inocula was inoculated with *H. gallinarum* grown as satellites to *Staphylococcus aureus* for 48 h on Casman blood-agar plates containing 5 % (w/v) citrated bovine blood. The MM contained (% w/v) in glass-distilled water: polypeptone (1.0), biosate (1.0), soluble starch (0.1) (Baltimore Biological Laboratories); beef extract (0.3) (Oxoid); nicotinamide (0.005),

p-aminobenzoic acid (0.005) (Calbiochem); dextrose (0.05), sodium chloride (0.9), Leptospira medium base EMJH (0.23) (Difco). A filter-sterilized solution of NADH (Calbiochem) was added before inoculation to give a final concentration of 0.0025 %.

The solution (WS) used for washing the organisms and dilution and/or adjustment of inocula for the increased CO₂ tension experiment contained (% w/v) in glass-distilled water: biosate (1.0), dextrose (0.05), soluble starch (0.1), thiamine-HCl (0.0005) (Nutritional Biochemicals Co.).

The test medium (TM) for the increased CO₂ tension experiment contained the same ingredients as the WS except that it was prepared in 0.02 M-Na₂HPO₄-KH₂PO₄ buffer using glass-distilled water containing 1.0 % (w/v) NaCl at pH 7.5 (PBS). Portions (4.5 ml) of TM were dispensed into optically-matched screw cap tubes (13 × 100 mm), and 0.5 ml of a filter-sterilized solution of NADH in PBS was added to each tube just before inoculation. The final NADH concentration was 0.025 mg/ml.

All media were adjusted to pH 7.5 with 1 M-NaOH and then autoclaved at 1.05 kg/cm² for 15 min before addition of NADH.

The chemically-defined medium contained (% w/v) in PBS: medium 199 (Difco), 1.1; Tween 80 (NBC), 0.05; and NADH, 0.0025. The medium was adjusted to pH 7.5 with 1 M-NaOH, filter-sterilized through 0.22 µm Millipore filter units and dispensed in 5.0 ml amounts into 16 × 150 mm tubes.

Bacterial cultures. *Haemophilus gallinarum* strains 17756, Modesto and 0222 were acquired from Dr R. Yamamoto, University of California, Davis, California, U.S.A., strains w and z from Dr J. Hanley, Pasco County Poultry Disease Diagnostic Laboratory, Dade City, Florida, U.S.A., and strain G was isolated by the authors from an outbreak of coryza near the University of Georgia.

Increased CO₂ tension experiment. Organisms grown in 20 ml of MM for 24 h were recovered by centrifuging and washed three times in equal volumes of WS before being resuspended to give a light transmittance of 90 %. Percentage transmittance (% T) was measured in the 13 × 100 mm tubes at 660 nm using a Bausch and Lomb Spectronic 20 spectrophotometer. Serial 10-fold dilutions of the adjusted suspension were made in WS and each dilution (using a single pipette) was inoculated into two tubes of TM and onto three Casman blood-agar plates using a 0.05 ml inoculum in each case. One tube of TM per dilution was incubated under aerobic conditions while the other was incubated under CO₂ tension. The inoculum on the Casman blood-agar plates was spread in an area approximately 2 cm square, surrounded by *S. aureus* and incubated under CO₂ tension for enumeration of colony-forming units (c.f.u.) according to the method of Page (1962).

Growth in each tube of TM was determined as less than 98 % T at 660 nm using a Spectronic 20 spectrophotometer. Measurements were made after 24 and 48 h incubation. Uninoculated media incubated under the same conditions served as standards and controls. All experiments were repeated three times.

Growth in defined media experiment. A 0.1 ml inoculum of organisms grown for 24 h in TM was used to colonize tubes of defined media. The organisms were maintained by serial passage of 0.05 ml inocula at 24 h intervals for seven such passages. Cultures were re-isolated from the seventh passage on Casman blood-agar plates cross-streaked with *S. aureus*. Uninoculated media incubated under the same conditions served as controls.

Culture conditions. All broth cultures (screw caps loosened) and agar plates were incubated for 24 and 48 h at 37 °C. Organisms grown in TM and defined media under CO₂ tension were incubated in No. 150 Gaspak jars (BBL). Approximately 5 % CO₂ tension was provided by three CO₂ generators (BBL) in each jar.

Table 1. *Effect of growing various strains of H. gallinarum in either CO₂ or an aerobic atmosphere at different dilutions*

A light transmittance of 98 % was considered to indicate 'no growth'. Each dilution was replicated three times for both CO₂ and aerobic atmospheres. 0 = undiluted, 1 = 10⁻¹, etc. The significance level is the probability that the proportion of tubes of *H. gallinarum* growing under CO₂ tension is greater than that of *H. gallinarum* growing aerobically. * The number of viable organisms necessary to initiate growth in a CO₂ atmosphere at 48 h. This was determined at the dilution showing growth in two or more tubes.

Growth in two or more tubes.										No. of viable organisms initiating growth*
Strain	Time (h)	Atmosphere	Dilution						Significance level	
			0	1	2	3	4	5		
Modesto	24	Aerobic	0	0	0	0	0	0	0.1	172
		CO ₂	3	2	0	0	0	0		
	48	Aerobic	1	0	0	0	0	0	0.005	
CO ₂		3	3	3	1	0	0			
W	24	Aerobic	0	0	0	0	0	0	NS	24
		CO ₂	2	0	0	0	0	0		
	48	Aerobic	3	0	0	0	0	0	0.05	
CO ₂		3	2	2	1	1	0			
17756	24	Aerobic	0	0	0	0	0	0	0.01	8
		CO ₂	3	2	0	0	0	0		
	48	Aerobic	3	0	0	0	0	0	0.005	
CO ₂		3	3	3	3	1	0			
G	24	Aerobic	0	0	0	0	0	0	0.01	18
		CO ₂	3	2	0	0	0	0		
	48	Aerobic	0	0	0	0	0	0	0.005	
CO ₂		3	3	2	0	0	0			
0222	24	Aerobic	0	0	0	0	0	0	0.025	2
		CO ₂	3	1	1	0	0	0		
	48	Aerobic	0	0	0	0	0	0	0.005	
CO ₂		3	3	3	3	1	0			
Z	24	Aerobic	3	3	3	2	2	0	0.05	0.68
		CO ₂	3	3	3	3	3	3		
	48	Aerobic	3	3	3	3	3	3	NS	
CO ₂		3	3	3	3	3	3			

NS, Not significant.

Statistical methods for the CO₂ experiment. The Fisher-Yates test of significance (Fisher, 1970) was utilized to determine whether *H. gallinarum* would grow as well or better in an increased CO₂ atmosphere as in an aerobic atmosphere. A table of significance was utilized to determine the meaning of the various differences in proportionality (Finney, 1948; Siegal, 1956).

RESULTS

The effect of increased CO₂ tension is given in Table 1. It appears that from approximately 1 cell (Z strain) to 172 cells (Modesto strain) were required to initiate growth in the TM under CO₂ tension. The viability of the organisms was not tested on other agar media. Our data only apply to growth in TM and to the parameters of the test conditions.

After 24 h, all strains except w grew significantly better (probabilities range from 0.01 to 0.05) in the CO₂ atmosphere. After 48 h all strains with the exception of z grew significantly better (probabilities range from 0.005 to 0.05) in the CO₂ atmosphere.

All the strains studied were capable of growth in the defined media. Although turbidity was apparent for each strain throughout the seven passages when compared to uninoculated controls, microscopic examination revealed mostly pleomorphic forms except in the case of strain z. These forms were predominantly long filaments with some coccoid bodies. Growth would probably have continued for more than seven passages. However, the experiment was terminated at this point, since the number of passages seemed sufficient for determining growth in the chemically-defined media.

DISCUSSION

Haemophilus gallinarum grew better in an increased CO₂ atmosphere as compared with an aerobic atmosphere. When grown under CO₂ tension in a broth medium lacking serum, fewer organisms were required to initiate growth, and the best growth occurred after 48 h in the medium tested.

It is probable that serum in a fluid medium provides a reduced environment immediately surrounding the organism, and that on an agar surface (even when serum is incorporated) the immediate environment is not reduced unless provided with a fixed atmosphere. Serum may provide additional nutritive substance(s) or act as a detoxifying agent. Evidence for serum serving as a source of other nutritive substance(s) could possibly be inferred from the studies of Delaplane, Erwin & Stuart (1938), who found that horse, sheep, goat, turkey and chicken sera supported growth of the organism while cow and pig sera did not.

It is well established that autoclaved artificial media may be toxic to some species of bacteria. Butler (1962) demonstrated that albumin and/or sodium oleate stimulated growth of *H. influenzae* and *H. parainfluenzae* in a chemically-defined media. It was postulated that albumin served as an adsorbent of growth inhibitors. Evans & Smith (1974) demonstrated that autoclaved proteose peptone was toxic to *H. influenzae* and that the toxicity could be neutralized on addition of dithionite. Delaplane *et al.* (1938) observed the potential value of agar as a growth factor for *H. gallinarum*. Agar is a detoxifier and a replacement for serum for other organisms (Hutner, 1942). As suggested by Page (1962), it is probable that serum acts as a detoxifier for growth of *H. gallinarum*.

Growth in the higher dilutions under CO₂ tension did not occur until 48 h. Possibly the test medium was somewhat toxic to the organisms and growth after 24 h was the result of an adaptive process. This delayed effect does not occur in the presence of 5 % (v/v) chicken sera (Rimler, unpublished).

Haemophilus gallinarum resembles other members of the genus *Haemophilus* in growing in a chemically-defined medium similar to that described by Talmadge & Herriott (1960) for *H. influenzae*. Growth in the defined media further confirms the lack of a requirement for haemin as demonstrated by Page (1962) and Biberstein, Mini & Gills (1963). The ability of all strains tested to grow in a chemically-defined medium through successive passages provides a mechanism for studying the nutritive requirements of the organism.

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