SHORT COMMUNICATION

Simplified Media for the Growth of *Haemophilus influenzae* from Clinical and Normal Flora Sources

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The nutritional requirements of 43 strains of *Haemophilus influenzae* isolated from clinical and normal flora sources were investigated. Two defined minimal media were developed by modifying the medium of Herriott *et al.* (1970): 74 % of the strains could grow on the minimal media and Herriott's medium; the remaining strains could not grow on any of these media.

INTRODUCTION

Kilian (1976) described five biotypes of *Haemophilus influenzae* based upon biochemical characteristics. This system has been used to correlate source of isolation and biotype (Bruun & Friis-Moller, 1976) and antibiotic resistance (Albritton *et al.*, 1978). However, the physiology of *H. influenzae* remains largely unknown. Several defined media have been described for the growth of *H. influenzae* (Butler, 1962; Talmadge & Herriott, 1960; Michalka & Goodgal, 1969; Herriott *et al.*, 1970; Wolin, 1963). The first four of these were developed for the growth of *H. influenzae* strain Rd, which is used for transformation studies (Alexander & Leidy, 1951). Only the medium of Herriott *et al.* (1970) could support the growth of the *H. influenzae* strains in our collection. Two modifications of that medium are reported here which simplify studies of the physiology of *H. influenzae* on defined media. These media have been used to further delineate the metabolic capabilities of *H. influenzae*.

METHODS

The growth of 43 strains of *H. influenzae* was examined: 11 of these were isolated as normal throat flora (from students at North Carolina State University), 30 were clinical strains (of which 25 were obtained from Dr Robert Weaver of the Center for Disease Control, Atlanta, Georgia, U.S.A.) and the two remaining strains (ATCC 19418 and ATCC 9795) were from the American Type Culture Collection.

The defined growth medium (M1C) was prepared according to Herriott *et al.* (1970); this medium was originally devised for the growth of *H. influenzae* strain Rd prior to the development of competence for transformation. The growth requirements of our strains on M1C were determined by omitting individual components from the medium in turn. Compounds tested for the ability to replace these were prepared in the manner of the constituent to be replaced. In this way, two modified media were devised: medium MMB contained ($\mu\text{g ml}^{-1}$) glutathione (200), glutamic acid (1300), L-arginine (300), uracil (100), inosine (2000), pantothenic acid (4), thiamin (4), NAD (4), haemin (10), K_2HPO_4 (3500), KH_2PO_4 (2700), NaCl (5800), MgCl_2 (430) and CaCl_2 (2.2); medium MMA was identical to MMB except that citrulline ($300 \mu\text{g ml}^{-1}$) replaced both arginine and uracil.

Broth cultures were incubated at 37 °C for 24 to 72 h on a New Brunswick rotary shaker at 200 rev. min⁻¹. The growth response was followed visually or measured turbidimetrically at 540 nm. Plates were incubated at 37 °C for 24 to 96 h in a jar with increased CO₂ tension (BBL GasPak carbon dioxide generator envelope).

Table 1. *Growth on minimal media of H. influenzae strains isolated from clinical sources and as normal throat flora*

Serotype	No. of strains	No. of strains able to grow within 72 h in:	
		MIC	MMA and MMB
<i>a</i>	8	7	7
<i>b</i>	18	16	16
<i>c</i>	2	1	1
<i>d</i>	3	3	3
<i>e</i>	3	0	0
<i>f</i>	2	0	0
Untypable	7	5	5

Capsule formation was detected by reaction with *Haemophilus influenzae* poly- and type-specific antiserum (BBL).

All biochemicals were from Sigma. Inorganic chemicals were of standard reagent grade.

RESULTS AND DISCUSSION

The nutritional requirements of four type *b* strains of *H. influenzae* were investigated initially, and two modified media (MMA and MMB) were devised by omitting components from MIC (see Methods). The minimal concentrations of arginine and uracil (MMB medium) and citrulline (MMA medium) which supported maximum growth in liquid culture were 75, 13 and 75 $\mu\text{g ml}^{-1}$, respectively. A direct growth response to glutamic acid and inosine at concentrations lower than those used in the media was noted.

Several constituents of MMA and MMB media could be replaced by other related compounds. The ability of citrulline to replace arginine and uracil (originally noted by Herriott *et al.*, 1970) indicates that *H. influenzae* is capable of pyrimidine biosynthesis under some conditions. Neither arginine nor uracil alone in the medium would support the growth of the strains. Ornithine could not replace arginine in the presence or absence of uracil. (This raises the question of the regulation of pyrimidine biosynthesis as modulated by these three compounds.) In the presence of arginine, uracil could be replaced by cytidine, 2-deoxycytidine or uridine but not cytosine, thymine or thymidine. Glutamic acid could be replaced by glutamine or 2-oxoglutarate but not proline or the intermediates of the tricarboxylic acid cycle. Glutathione or cysteine but not methionine could replace cystine. Glutathione promoted the most luxuriant growth and was included in the minimal media for this reason.

Growth of the *H. influenzae* strains on MMA and MMB media solidified with 1.25 % (w/v) agar (Difco Noble Agar) was enhanced by the incorporation of bovine serum albumin. Growth was also higher when arginine, uracil and citrulline were present at the concentrations stated in Methods, rather than at the minimal concentrations for maximum growth in liquid cultures. Tween 80 and polyvinylalcohol have been reported to enhance the growth of *H. influenzae* by absorbing toxic products and metabolites in the medium (Talmadge & Herriott, 1960; Wolin, 1963); a similar role was proposed for bovine serum albumin by Butler (1962). We found that these compounds enhanced the growth of our strains on solid media and so we included them (Tween 80 and polyvinylalcohol at 20 $\mu\text{g ml}^{-1}$; bovine serum albumin at 1000 $\mu\text{g ml}^{-1}$).

The minimal media MMA and MMB supported the growth of 32 of the 43 *H. influenzae* strains investigated (Table 1) and two strains of *H. parainfluenzae*, but not the growth of one strain of *H. haemolyticus*. However, if these media are used for isolation purposes, other *Haemophilus* species may be isolated. The 11 strains of *H. influenzae* unable to grow in MMA or MMB also failed to grow in MIC and therefore did not simply require one of the

additional amino acids or other constituents of that medium. No strains were found that grew in only one minimal medium. Each strain required glutathione, glutamic acid, arginine, uracil and inosine for growth. Arginine and uracil could be replaced by citrulline for each strain.

The minimal media MMA and MMB derived from MIC define more specifically the growth requirements of a large number of *H. influenzae* strains encompassing all six serotypes and untypable strains. Over 90 % of the type *b* strains investigated were able to grow in MMA and MMB. Serotype *b* is the most common serotype isolated from *H. influenzae* infections (Kilian, 1976). Our results indicate that the nutritional requirements for glutamic acid, glutathione, inosine and citrulline (or arginine and uracil) may be common in *H. influenzae* strains. We therefore feel that these two minimal media will be useful for the elucidation of the physiology of *H. influenzae*.

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