The Effect of Sodium Chloride and NADH on the Growth of Six Strains of *Haemophilus* Species Pathogenic to Chickens

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SUMMARY

Six strains of *Haemophilus* species, pathogenic to chickens, required 1.0 to 1.5 % (w/v) NaCl for optimum growth. The requirement was for Na⁺ rather than NaCl. A sodium salt buffer influenced the optimum NaCl requirement and enhanced growth. Each strain required a different concentration of NADH for an optimum rate of growth.

INTRODUCTION

Evidence has been presented that *Haemophilus gallinarum* utilizes NAD (V factor) rather than both X factor (haemin) and V factor as a supplement for growth (Biberstein, Mini & Gills, 1963; Page, 1962). This finding was contrary to earlier descriptions by Schalm & Beach (1936) and Delaplane, Erwin & Stuart (1938), who reported requirements for both X and V factors. Because of discrepancies between these nutritional studies, Biberstein & White (1969) proposed a classification for two distinct species of *Haemophilus* which cause infectious coryza of chickens. The proposed species were *H. gallinarum* and *H. paragallinarum*.

According to this classification, *H. gallinarum* requires both X and V factors, and approximately 1.0 % NaCl for optimum growth. In contrast, *H. paragallinarum* requires only V factor with not more than 0.5 % NaCl. Both species require an increased CO₂ tension with the addition of serum to the medium (Zinnemann & Biberstein, 1975).

Strains of the organism originally described as *H. gallinarum* (Delaplane, Erwin & Stuart, 1934; Elliot & Lewis, 1934; Nelson, 1932; Schalm & Beach, 1936), and reported to require both X and V factors, have been lost and are not available for study. The requirement of X factor for these strains is questionable.

Considering the possible existence of two distinct species of *Haemophilus* which cause an infectious coryza of chickens, the current descriptions of differential characteristics may be accurate only with respect to the X and V factor requirement. Only the original *H. gallinarum* strains were studied with regard to optimum NaCl concentration and requirement (Delaplane *et al.*, 1938; Gregory, 1944). Although a requirement for NaCl was established, differences in optimum concentrations were observed. Extension of these studies raised doubt as to a need for serum for the growth of these strains (Gregory, 1944).

The objective of our study was to define the NaCl and NADH requirements of six strains of haemophilic Gram-negative bacteria which do not require the X factor, and which cause infectious coryza of chickens.

METHODS

Bacterial cultures. The six strains described by Rimler *et al.* (1976) were used, of which five were designated *H. gallinarum* at acquisition.

Slide agglutination using type-specific *H. gallinarum* antiserum acquired from Dr L. A. Page, National Animal Disease Center, Ames, Iowa, U.S.A., demonstrated that strains Modesto, w and 17756 were serotype A. Strain 0222 was serotype B and strain z was serotype C. Strain G reacted to all the typing sera.

Initially all strains were pathogenic for chickens, but laboratory manipulation has probably rendered strains 0222 and z avirulent.

Media. The maintenance medium (MM), the method used for maintenance of cultures, preparation of inocula, and the solution (WS) used for washing the organisms and adjustment of inocula density were as described by Rimler *et al.* (1976).

The test medium base (TMB) contained the same ingredients as WS. All test media were adjusted to pH 7.5 with 1 M-NaOH, dispensed in 5 ml portions in 13 × 100 mm optically matched screw cap tubes and autoclaved at 1.05 N cm⁻² for 15 min unless stated otherwise. NADH (Calbiochem) was sterilized by filtration through 0.22 µm Millipore filter units and added aseptically when required.

Culture conditions. All broth cultures (screw caps loosened) were incubated for 24 and 48 h at 37 °C under CO₂ tension as previously described (Rimler *et al.*, 1976).

Standardization of inocula and measurement of growth. Organisms grown in 20 ml MM for 24 h were recovered by centrifuging, washed three times in equal volumes of WS, and resuspended to give a light transmittance (T) of 90 %. An inoculum of 0.05 ml per tube of this suspension was used to seed all test media. Inocula were standardized and growth was measured turbidimetrically in the 13 × 100 mm tubes at 660 nm. TMB served as the standard for adjustment of inocula; uninoculated control tubes served as standards for measurements of growth. Measurements were made after 24 and 48 h incubation. All experiments were repeated three times.

Effect of NaCl and other salts on growth. The effect of NaCl from 0 to 3.0 % (w/v) was tested in both TMB and TMB prepared in 0.02 M-phosphate buffer pH 7.5. Other salts examined were NaH₂PO₄, KH₂PO₄ and MgSO₄ at 0.085 and 0.17 M. Where necessary the pH was adjusted to 7.5 with 1 M-NaOH. All media contained 50 µg NADH ml⁻¹.

Optimum NADH requirement. This requirement was examined in TMB prepared in 0.02 M-phosphate buffer pH 7.5 and containing 1 % (w/v) NaCl. NADH was added to give a twofold incremental sequence in final concentration covering the range 1.56 to 100 µg ml⁻¹. Medium without NADH was also included.

Statistical analysis. The effect of NaCl on the growth of individual strains after 48 h incubation was examined in an initial analysis of variance using Duncan's New Multiple Range Test (Steel & Torrie, 1960). 'No growth' was interpreted as 100 % T in two replicates and 99.5 % T or greater in the third. A factorial analysis was done for the 48 h growth period because the results were more definitive. The factors considered were: (i) strain; (ii) NaCl medium (with or without buffer) and (iii) percentage NaCl (with or without buffer) over the 0.5 to 1.5 % range. A final two-factor analysis of variance was calculated for both incubation periods to make single degree of freedom comparisons (orthogonal).

The data for NADH requirement were analysed for each incubation period using an

Fig. 1. Growth of six strains (Modesto, w, 17756, G, 0222, Z) of *Haemophilus* species in TMB with and without buffer and NaCl.

Table 1. *Orthogonal comparisons* of the mean† light transmittance of six strains of Haemophilus species grown in TMB, with and without buffer, and containing different concentrations of NaCl*

Strain	Growth at 24 h (%T)						Growth at 48 h (%T)					
	NaCl (% w/v) alone			NaCl (% w/v) with buffer			NaCl (% w/v) alone			NaCl (% w/v) with buffer		
	0.5	1.0	1.5	0.5	1.0	1.5	0.5	1.0	1.5	0.5	1.0	1.5
	A	B	C	D	E	F	A	B	C	D	E	F
Modesto	98.1	78.8	80.5	83.8	76.2	80.7	89.3	84.0	85.5	82.0	82.8	83.0
w	99.5	81.8	86.0	84.8	76.8	89.8	89.2	81.8	84.3	78.5	78.8	80.0
17756	92.0	80.0	82.8	79.7	78.0	86.5	81.8	79.2	77.0	74.5	74.7	78.3
G	99.0	84.5	91.2	85.5	83.0	96.2	95.2	86.8	87.3	77.8	79.5	80.7
0222	85.0	79.5	78.8	81.3	77.0	81.0	85.3	78.3	79.7	78.5	77.0	82.3
Z	70.2	75.0	74.2	64.7	66.5	65.3	75.5	72.0	73.7	66.2	65.0	68.3
Analysis	Significance level						Significance level					
ABC v. DEF	> 0.005						> 0.01					
A v. BC	> 0.05						> 0.005					
F v. DE	NS						> 0.01					
B v. C	NS						NS					
D v. E	NS						NS					
B v. E	> 0.05						> 0.025					
D v. F	NS						> 0.005					
C v. F	> 0.05						NS					
E v. F	> 0.05						> 0.05					

NS, Not significant.

* Four sets of comparisons, extraneous data not included. Since the error components were heterogeneous (Bartlett's chi-square test for homogeneity) each comparison was tested using its own error component.

† Three trials.

Table 2. *Number of replicates (out of a total of three) showing growth (< 98 % T at 660 nm) of six strains of Haemophilus species in TMB with NaCl replaced by another salt*

Salt	Time (h)	Cation concn (M)	Strain					
			Modesto	w	17756	G	0222	Z
NaH ₂ PO ₄	24	0.085	3	2	3	3	3	3
		0.17	2	0	2	0	1	3
	48	0.085	3	3	3	3	3	3
		0.17	3	3	3	2	3	3
KH ₂ PO ₄ } K ₂ HPO ₄ }	24	0.085	0	0	0	0	0	0
		0.17	0	0	0	0	0	0
	48	0.085	0	0	0	0	0	0
		0.17	0	0	0	0	0	0
MgSO ₄	24	0.085	0	0	0	0	0	3
		0.17	0	0	0	0	0	0
	48	0.085	0	0	0	0	0	3
		0.17	0	0	0	0	0	0
NaCl control	24	0.085	3	3	3	3	3	3
		0.17	3	3	3	3	3	3
	48	0.085	3	3	3	3	3	3
		0.17	3	3	3	3	3	3

* pH was adjusted to 7.5 using K₂HPO₄ rather than NaOH.

Table 3. *NADH requirement for growth of six strains of Haemophilus species*

Strain	Time (h)	Optimum NADH concn for maximum growth* ($\mu\text{g ml}^{-1}$)
Modesto	24	3.13
	48	≤ 1.56
w	24	12.5
	48	12.5
17756	24	12.5
	48	6.25
G	24	25
	48	12.5
0222	24	6.25
	48	≤ 1.56
Z	24	≤ 1.56
	48	≤ 1.56

* The growth response for each strain to an NADH concentration greater than these values was not significantly different at the 5 % level of probability.

analysis of variance (two-way analysis with one observation/cell). Each mean value was compared with every other mean value using Tukey's W procedure (Steel & Torrie, 1960).

RESULTS

Effect of NaCl on growth. Generally growth was best in both media within the 0.5 to 1.5 % NaCl range. Within this range the strains, with the exception of 0222, grew better in the buffered medium (Fig. 1). The detailed results with an analysis are given in Table 1.

Replacement of NaCl with other salts. Replacement of NaCl with any salt other than a sodium salt resulted in the complete absence of growth in every case, with the exception of strain z and MgSO_4 . However, the incidence of growth at 24 h with 0.17 M- NaH_2PO_4 was less than with 0.17 M-NaCl suggesting that the high phosphate molarity may be detrimental (Table 2).

Optimum NADH requirement. The optimum concentration of NADH for maximum growth of all strains at 24 h ranged from 1.56 to 25 $\mu\text{g ml}^{-1}$ and at 48 h this requirement was reduced for at least five of the six strains (Table 3). No growth occurred in the absence of NADH.

DISCUSSION

Previously, the six strains of coryza-producing organisms used would have been regarded taxonomically as *Haemophilus paragallinarum*. This and a previous study (Rimler *et al.*, 1976) have shown that although these strains require only the V factor, the other growth requirements demonstrated are compatible with descriptions found in the literature for *Haemophilus gallinarum* (Delaplane *et al.*, 1938; Gregory, 1944).

The concentrations of NaCl required for optimum growth were between 1.0 and 1.5 % (w/v) confirming those reported by Gregory (1944). Delaplane *et al.* (1938) reported an NaCl optimum between 1.5 to 2.0 % (w/v); however, their method for determination of this requirement employed broth at the base of agar slopes which may partly account for the variations in the optimum NaCl range observed.

Inclusion of a sodium salt buffer into a broth medium provides better growth potential and apparently contributes to the sodium requirement of the organism. This contribution of sodium ions by the buffer may explain the ability of some strains to grow in 0.5 % NaCl at 24 h. The decrease of this optimum range at 48 h for the same organism in buffer to between 0.5 to 1.0 % NaCl may be due to the presence of phosphate. In any event the best growth for all strains was obtained in a buffered medium containing 1.0 % NaCl.

There is not an absolute requirement for NaCl in this system since it could be replaced by another sodium salt, though not by potassium or magnesium salts. Growth was less with sodium phosphate than with NaCl, especially at the higher concentration. This may be due to the high phosphate concentration or the lack of chloride.

All six strains studied required V factor (NAD). Growth varied with different concentrations and strains. In a study of the NAD requirement of *H. parainfluenzae*, Evans, Smith & Wicken (1974) also observed variation of the optimum levels within this species. It is interesting that the highest optimum at 24 h growth of the six strains studied here (25 $\mu\text{g ml}^{-1}$) was the same as that noted in their study. Most apparent in our study was the lowered optimum level required for maximum growth at 48 h; several strains only required NADH at about the minimum of 2.5 $\mu\text{g ml}^{-1}$ reported by Page (1962). The decrease in requirement for NADH after 24 h in this case probably reflects a difference in the growth rate of the respective strain. If this is true, higher concentrations of NADH (to a certain optimum level) will produce a greater growth rate. Differences in V factor requirements could explain previous reports of maximum growth at 24 h.

All strains grew better in an increased CO_2 atmosphere than in an aerobic atmosphere (Rimler *et al.*, 1976).

From our studies it would appear that the characteristic properties for differentiation of *H. gallinarum* and *H. paragallinarum* should be confined to observed X and V factor variations and should not include NaCl requirements.

Recent reports by Evans & Smith (1972) and Evans *et al.* (1974) have emphasized the

difficulty encountered when impure compounds are used as sources of X factor for *Haemophilus* speciation. Evans & Smith (1972) reported that 11 of 37 V factor-dependent strains of human origin required an additional factor present in horse blood and thus suggested a requirement for both X and V factors. This assumption was shown to be invalid when sodium oleate was used instead of blood and the resultant growth was easily characterized as *H. parainfluenzae*.

The ability of *Haemophilus* species to synthesize their own X factor does not preclude the enhancement of growth when this factor is available from an exogenous source. This rather confusing characteristic is most likely associated with the use of impure compounds which provide sufficient amounts of haemin intermediates to permit growth. Such phenomena have been documented for *H. parainfluenzae* (Evans *et al.*, 1974) and for certain of the *pasteurellae* (Jordan, 1952).

It was not within the scope of our current protocol to prove the need or lack of an X factor requirement for the *H. gallinarum* strains of Schalm & Beach (1936) and Delaplane *et al.* (1938). However, such proof would relate directly to their use of horse blood or its extracts as sources of X factor.

Recent studies in our laboratory have shown that an oleic-albumin complex can partially replace the chicken serum requirement for the growth of our strains (Rimler, unpublished). In view of these data and the previous similar documentation, it is reasonable to challenge the absolute X factor requirement reported for the original strains of *H. gallinarum*.

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