The Effect of Osmotic Variation upon the Growth of *Vibrio fetus*

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SUMMARY

The growth rate of *Vibrio fetus* in laboratory media could be stimulated or inhibited by the addition of appropriate concentrations of widely different potential nutrients and salts. The effect appeared to depend on the osmotic activity of the supplements since, with electrolytes, growth was maximal at about the same osmolal concentration irrespective of the chemical nature of the electrolyte. With sucrose a higher osmolality was required. In comparative tests the growth rate of *Escherichia coli* was much less affected by variation in solute concentration. *V. fetus* appears unusually sensitive to change in the osmolality of the growth medium and this may account for the general difficulty of obtaining satisfactory growth of the organism.

INTRODUCTION

Since the time of its first isolation from infected animals by McFadyean & Stockman (1913) and Smith (1918), *Vibrio fetus* has proved difficult to grow in laboratory media. A variety of complex and synthetic media has been used by different workers with varying degrees of success (Arne Hansen, Price & Clements, 1952; Brinley Morgan, 1957; Trueblood & Tucker, 1957; Jakovljevic & Beattie, 1960; Zemjanis & Hoyt, 1960; Fletcher & Plastringe, 1963; Smibert, 1963; Garvie, 1967; Fung & Winter, 1968) but the optimal physicochemical conditions for growth have not yet been adequately defined (Tritz & Ogg, 1967a). During investigation of a possible nutritional basis for the placental localization of *V. fetus* (Lowrie & Pearce, 1970) the growth rate in laboratory media was affected by an unusually wide range of compounds – suggesting that the materials were not acting simply as nutrients. This paper describes the effect of variation in osmotic activity on the growth of *V. fetus*.

METHODS

Organisms. *Vibrio fetus* var. *intestinalis* strain Berryman and *Escherichia coli* K12 were grown at 37 °C under 10 % CO₂ in air in a nutrient broth (see below). Inocula for medium supplementation experiments were taken from the late logarithmic phase of growth and, after twice washing the organisms in phosphate buffered saline, pH 7.4 (0.70 g KH₂PO₄, 2.13 g Na₂HPO₄, 7.50 g NaCl, to 1 l with double-distilled water), the concentration of the final suspension was adjusted so that 0.1 ml, when added to 0.9 ml of medium, gave about 1 to 5 × 10⁴ colony forming units (c.f.u.)/ml (Lowrie & Pearce, 1970).

Media. For supplementation studies a nutrient broth (Lowrie & Pearce, 1970) was made up at double strength but without addition of NaCl. A saline extract of ovine muscle was prepared as previously described (Lowrie & Pearce, 1970) but the NaCl content was then approximately doubled by the addition of 10 % (v/v) of 1.5 M-NaCl. After twofold dilution

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with either distilled water or experimental supplement, the NaCl concentration was at the optimum for growth as determined in preliminary studies.

Media supplements. Foetal cotyledon diffusate was prepared as previously described (Lowrie & Pearce, 1970) except that tissue was macerated in distilled water before dialysis. The defined compounds used as supplements were supplied by BDH Chemicals Ltd, Poole, Dorset; all were analytical grade except mono-sodium glutamate. Solutions of known concentration were prepared in distilled water, sterilized by membrane filtration (0.22 μ m pore size, Millipore (UK) Ltd, London NW10 7SP) and doubling dilutions in sterile distilled water prepared as stock for medium supplementation.

Assay of growth enhancement. Growth enhancement on addition of supplements to media was assessed in one of two ways. In the majority of experiments viable numbers were measured as c.f.u. at a single time (24 h for *Vibrio fetus*, 4 h for *Escherichia coli*) after inoculation; growth enhancement was expressed as c.f.u./ml of supplemented medium divided by c.f.u./ml of unsupplemented medium. In certain experiments growth was measured by c.f.u. count at intervals during 24 h, so that the effect of supplementation on duration of the lag phase and the rate of growth could be determined.

Estimation of osmolality. Growth media and each stock dilution of supplement were diluted 1 in 2 with water and measurements of final osmolal concentration (m-osmoles/kg water) made using a freezing point osmometer (model 63-31, Advanced Instruments Inc., Massachusetts, U.S.A.). Standard NaCl solutions were used for instrument calibration.

The osmolality of each supplemented medium was approximated as the sum of the osmolality of the twofold diluted medium and that of the diluted supplement solution.

RESULTS

Enhancement of growth of Vibrio fetus in a muscle extract medium

Lowrie & Pearce (1970) showed that extracts of ovine muscle supported a lower growth rate of *Vibrio fetus* (population doubling time 3 to 6 h) than similar extracts of ovine placental tissue. Muscle extract medium was therefore regarded as suitable for the detection of the growth factors presumed to be present in placental extracts. However, not only foetal cotyledon diffusate but also any of a range of organic and inorganic supplements, with the exception of NaCl, enhanced growth at 24 h when added at appropriate concentrations (Table 1). Stimulation of growth increased with increased supplement concentration to a maximum where the growth-rate approximated that in a routine laboratory medium such as nutrient broth containing 10 % (v/v) horse blood (doubling time about 2 h). Beyond this optimum supplement concentration growth enhancement was progressively decreased (Fig. 1); at high concentrations all supplements were inhibitory. The degree of growth enhancement observed with a given supplement varied between replicate experiments in which different inoculum preparations were used; the optimum concentration was constant.

The variety of materials with growth-enhancing activity suggested that the supplements did not exert their effect by direct participation in cellular metabolism. When the osmolal concentrations of the supplements were determined by freezing point measurement the optimum supplement osmolality for growth was found to be between 30 and 60 milliosmoles with electrolytes and rather higher with sucrose (Table 1). It was possible with some of the supplements, especially with the foetal cotyledon diffusate, that part of the growth-enhancing effect was due to nutrient stimulation. However, when the effect of addition of foetal cotyledon diffusate alone was compared with addition of diffusate and NH_4Cl together, the same growth enhancement was observed at the same osmolality, regardless

Fig. 1. The effect on growth of increasing the osmolality of muscle extract medium from 284 milliosmoles by the addition of foetal cotyledon diffusate (○) or NH_4Cl (▲). Growth enhancement was calculated as the ratio of c.f.u./ml of supplemented medium to c.f.u./ml of unsupplemented medium, at 24 h.

Table 1. Supplement concentrations for maximum growth rate of *Vibrio fetus* in ovine muscle extract medium

Supplement	Concentration in medium	
	mg/ml	Osmolality* (milliosmoles)
Diffusate†	3.0	46
NH_4Cl	1.5	56
Sodium acetate	1.3	31
Sodium glutamate	4.6	51
NaCl	NI	NI
Na_2SO_4	2.4	41
KCl	1.7	51
Sucrose	40	116

Growth enhancement was tested for each supplement over a range of concentrations in at least two experiments. Concentration optima were estimated graphically by plotting growth enhancement against the osmolality of the supplemented medium; typical values are shown.

NI, no increase: increase in concentration above 7.5 mg/ml (the basal level in muscle extract) decreased the growth rate.

* Determined by freezing-point measurement using NaCl as a standard.

† Foetal cotyledon diffusate was prepared by dialysis of macerated tissue in distilled water.

of the ratio of the two supplements (Fig. 2). The two growth enhancement curves would not have been expected to superimpose had there been a difference in the mechanism of action of the two supplements. Failure to demonstrate in this way a nutritional effect of the cotyledon diffusate accorded with results of diffusate fractionation studies. Fractions prepared by gel-filtration, ion-exchange and paper chromatography all showed some degree of growth

Fig. 2. The equivalence in growth enhancement, in muscle extract medium, achieved by supplementation with foetal cotyledon diffusate alone (○) or with a constant amount of NH_4Cl (0.67 g/l; 24 milliosmol) and various amounts of diffusate (●). Growth enhancement was calculated as described for Fig. 1.

enhancement; furthermore, the activity of each fraction could be related to its osmolality. It was concluded that an osmotic effect of added supplements was responsible for growth enhancement.

With population size routinely measured only once after inoculation, the growth enhancement could have been due to a decrease in a lag period rather than to increase in growth rate. Viability measurements at intervals after inoculation showed that the main effect of alteration of the osmolality of the supplemented muscle extract was upon growth rate (Fig. 3).

Growth studies in nutrient broth

The osmotic sensitivity of *Vibrio fetus* was also manifested in a standard medium, indicating that the phenomenon was not dependent on some abnormal feature of the muscle extract medium. Nutrient broth supplemented (10 %, v/v) with any of the materials listed in Table 1 showed no significant growth enhancement but growth was decreased with increased supplement concentration. In addition, when the broth was prepared without the usual content of NaCl (5 g/l) and to this medium different concentrations of supplement were added, growth enhancement was observed (Fig. 4). Growth increased with osmolality and then decreased in a manner comparable to that observed with muscle extract. Measurement of growth at intervals after inoculation showed, as with muscle extract medium, that alteration in osmolality affected growth rate and not duration of lag phase.

Fig. 3. Growth rates in muscle extract medium, supplemented either with foetal cotyledon diffusate (○) or NH_4Cl (▲) to an osmolality (medium plus supplement) of 310 milliosmoles, or unsupplemented (×).

Comparison with *Escherichia coli*

The pronounced sensitivity of *Vibrio fetus* to change in the osmolality of the medium was thought to be unusual amongst bacteria. The effect upon the growth of *Escherichia coli* in nutrient broth (Fig. 4) was, in contrast, much less marked.

DISCUSSION

A large decrease in bacterial growth rate as a result of a relatively small shift from optimum solute concentration, as described here, seems unusual; certainly the growth rate of *Escherichia coli* was less affected in our tests. In accord with early observations on the effect of salts on growth of *E. coli* (Sherman, Holm & Albus, 1922), the major effect of variation in the supplement concentration in the medium was on the rate of growth during the logarithmic phase. Although bacterial sensitivity to solute concentration is well known, Gram-positive species being tolerant to greater extremes than Gram-negative species (Mitchell & Moyle, 1956), comparable studies of the effect upon the growth rates of other Gram-negative species have not been made. Reports indicate that a similar degree of sensitivity might be found among those organisms which, like *Vibrio fetus*, show delicate growth in laboratory media (Miller, Hastings & Castles, 1932; Hardy & Nell, 1961; Grund & Förster, 1969).

An osmotic basis for the sensitivity of *Vibrio fetus* to solute concentration was indicated by the similarity of the osmolal concentrations of the different solutes which resulted in maximum growth rate. Although the osmolalities required for maximum growth rate were merely similar and not identical, the effect may, nevertheless, be predominantly an osmotic one since bacterial membranes exhibit different permeability to different substances.

Fig. 4. The effect of change in osmolality of nutrient broth on the growth of *Vibrio fetus* (□) and *Escherichia coli* (■). Nutrient broth prepared without NaCl (97 milliosmolal) was supplemented with varying concentrations of NaCl. Growth enhancement was determined 24 h after inoculation with *V. fetus* or 4 h after inoculation with *E. coli* (calculation as described for Fig. 1).

Direct measurements of osmotic pressure (Weibull, 1955; Mitchell & Moyle, 1956) might have shown that at optimum osmolality the osmotic pressure across the microbial envelope was the same regardless of the nature of the solute. In addition, other effects of solutes upon the organism could contribute to the observed differences. Direct involvement in cellular metabolism or in the mechanical stability or functional integrity of the bacterial envelope is possible (McQuillen, 1960) since studies of spheroplast stabilization have shown a comparable dependence of solute concentration optima upon the nature of the osmotically active material (Weibull, 1956; Britten, 1965; Montgomerie, Kalmanson & Guze, 1967). Failure to obtain maximum growth rate of the organism by supplementing exclusively with NaCl in either muscle extract or NaCl-free nutrient broth (D. B. Lowrie, unpublished) is probably due to a toxicity of NaCl at osmolal concentrations which would otherwise result in maximum growth rate; susceptibility to NaCl toxicity is characteristic of the species (DiLiello, Poelma & Faber, 1959).

The mechanisms by which variation in osmotic pressure might influence the growth rate of *Vibrio fetus* are not known. Enhancement of growth rate of the organism with increased osmolality may have been a result of increased bacterial division rate, decreased death rate, or both. The possibility of an effect on division rate is indicated by the finding that respiration rate in intact bacteria was increased by increased solute concentrations; above critical concentrations respiration rate decreased (Knowles & Smith, 1971); alterations in membrane structure were probably involved. Decrease in death rate with increased osmolality could occur through stabilization of an osmotically fragile proportion of the growing population against lysis under osmotic pressure or against lethal metabolic disruption.

Circumstantial evidence that such organisms are produced by *V. fetus* is provided by the frequent appearance of spherical forms during laboratory cultivation (Ogg, 1962; Tritz & Ogg, 1967*b*). Hardy & Nell (1961) demonstrated that the appearance of similar forms in cultures of treponemes was a consequence of osmotic imbalance between the organisms and their environment and that the spherical forms were not viable. Osmotic imbalance during the growth of an osmotically-sensitive mutant of *Salmonella typhimurium* was shown to result, first in the appearance of spherical forms and secondly in loss of viability (Antón, 1972).

Recognition of the importance of the osmolality of the medium for the growth of *Vibrio fetus* calls into question earlier proposals, based upon growth rate differences in a range of tissue extracts, on the presence of a growth factor in ovine placenta and its possible role in placental localization (Lowrie & Pearce, 1970). Measurements on such extracts revealed high osmolalities and indicated that differences in osmotic activity could not account for the superiority of placental extracts over extracts of other tissues (D. B. Lowrie, unpublished); hence a nutritional basis for high growth rate in placental extract remains a possibility. Another explanation might be that some extract components exert a protective effect against high solute concentration. Several constituents of yeast extract, one of which was identified as betaine, were found to protect *Vibrio percolans* against media of high osmolality (Dulaney, Rickes & Dulaney, 1967).

The difficulty of growing *Vibrio fetus* in the laboratory could stem from the sensitivity of the organism to osmotic imbalance and not from a requirement for unusual metabolites. This interpretation is supported by our failure to find any growth stimulatory effect of added nutrients which could not be accounted for as an osmotic effect. Attention to the nature and concentration of the osmotically active components of media formulated for the growth of *V. fetus* may lead to improved media for routine use.

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