Determination of Some *in vitro* Growth Requirements of *Bacteroides nodosus*

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(Received 21 August 1974; revised 30 September 1974)

SUMMARY

Physical and nutritional factors required for growth of *Bacteroides nodosus* isolates from ovine foot-rot lesions were examined. Simplified anaerobic culture techniques were devised utilizing a fully soluble, autoclavable, liquid medium (TAS) which contained proteose-peptone, yeast and meat extracts and certain other essential compounds required to promote prompt and serially transferrable growth of cultures from small inocula. The latter included Trypticase, arginine, a reducing agent (most suitably thioglycollic acid) and CO₂; serine and Mg²⁺ markedly increased growth yields. Trypticase could not be replaced by a commercial preparation of acid-hydrolysed casein; other forms of hydrolysed proteins gave delayed and inconsistent growth. Maximum growth of cultures required concentrations of 0.02 to 0.05 M-arginine, which could not be replaced by glutamic acid, citrulline or ornithine. Exogenous carbohydrate compounds were not required.

The temperature range for optimum growth of cultures was 37 to 39 °C, and anaerobic culture conditions were essential for growth and the production of *B. nodosus* organisms of normal morphology. Solidified TAS media for the isolation and maintenance of *B. nodosus* cultures were also devised.

INTRODUCTION

Bacteroides nodosus (*Fusiformis nodosus*), a Gram-negative, non-sporeforming anaerobe, has been recognized as the principal causative agent of foot-rot in sheep, a chronic disease that develops as a mixed bacterial infection of basal epidermal layers of the hoof (Beveridge, 1941; Egerton, Roberts & Parsonson, 1969). As complex methods are required to cultivate strains *in vitro*, little biochemical study of this organism has been attempted.

Originally, Beveridge (1941) used a special agar medium containing horse serum for isolating and maintaining cultures, but not all batches of serum were satisfactory and growth was poor in a liquid medium of similar composition. Subsequent attempts to isolate *B. nodosus* by this method were unsuccessful (Beveridge, 1956; MacKenzie, 1956; Thomas, 1958); however, Thomas (1958) eventually obtained consistent growth of cultures on a serum-free agar medium supplemented with finely ground horn from sheep's hooves. A liquid medium incorporating a reducing agent, acid-hydrolysed horn or wool, and auto-digested pancreas or trypsin was also claimed to support significant growth of cultures in 3 to 5 days under nitrogen (Thomas, 1963).

Modifications of Thomas's media include enrichment with yeast and liver extracts, bovine blood, amino acids and various protein digests (Parsonson, Egerton & Roberts, 1967; Marsh & Claus, 1970; Egerton, 1974), and the combining of autoclaved extracts

of hoof and of trypsin in a liquid medium (Roberts, 1967) and in a biphasic medium (Egerton & Burrell, 1970). These culture milieux portrayed *B. nodosus* as a fastidious heterotroph, seemingly uniquely adapted for localized growth in hoof epidermal tissue. However, Walker, Short, Thomson & Roberts (1973) reported that two strains of *B. nodosus* thrived in a comparatively simple medium of peptone and yeast extract, although consecutive cultures of these organisms evidently required large inocula (10 %, v/v) to produce the bacterial yields claimed.

Because a physiological characterization of *B. nodosus* strains was contemplated, autoclavable, non-precipitating media yielding prompt and reproducible growth were desired. Furthermore, since *B. nodosus* adjuvant vaccines have prophylactic and therapeutic effects on ovine foot-rot (Egerton & Burrell, 1970), potentially large-scale methods were required for producing cultures of high biomass concentration and immunogenicity. This paper describes an investigation of various growth determinants of *B. nodosus* leading to the derivation of simplified media that support improved growth of serially transferred, virulent cultures from small inocula. A description of the liquid medium developed has been published previously in the industrial patent literature (Skerman, 1972).

METHODS

Organisms. Preliminary experiments were carried out with *B. nodosus* strains isolated on hoof agar (Thomas, 1958). Cultures were also supplied by J. R. Egerton, McMaster Laboratory, C.S.I.R.O., Sydney, Australia. Fresh isolates, obtained by using modified media, were included in later experiments and were used to verify previous results. All strains were conserved by freeze-drying bacteria harvested from agar cultures. Stock cultures, subcultured weekly, were grown on agar for 4 to 5 days and then stored under hydrogen at room temperature for up to 14 days.

Medium constituents. Proteose-peptone, trypsin 1:250, Bacto-Casitone, Bacto-Tryptone Bacto-Casamino acids, Bacto-Oxgall and Bacto-yeast extract were from Difco, 'Lab-Lemco' beef extract, liver digest and sodium thioglycollate were from Oxo Ltd, London; Trypticase, and the peptones Lactalsate, Thiotone, Phytone, Gelysate and Myosate, were from BBL Division of Bioquest, Becton, Dickinson and Co., Maryland, U.S.A.; amino acids, haemin, reduced glutathione, menadione, dithiothreitol and thioglycollic acid (95 % aqueous) were from Sigma; sodium formaldehyde sulfoxylate and all inorganic chemicals used were from BDH; agar was supplied by Davis Gelatin (New Zealand) Ltd, Christchurch, New Zealand.

Carbon dioxide, nitrogen and $\text{CO}_2 + \text{N}_2$ gas mixtures were passed through a deoxygenating column of heated copper turnings before use (Moore, 1966).

Hoof powder was prepared as outlined by Thomas (1958). Acid or alkaline hydrolysis of 200 mg quantities of hoof powder was carried out in sealed ampoules as described by Brenner, Niederwieser & Pataki (1965). Hoof powder diffusate was obtained by dialysing a suspension of 2 g powdered hoof in 20 ml water against 80 ml distilled water for 7 days at 4 °C.

Aqueous extracts of trypsin, hoof powder, and heat-killed *B. nodosus* harvested from broth cultures were each prepared by autoclaving 1 g dry wt in 100 ml distilled water at 121 °C for 10 min and removing the insoluble components by filtration.

Sheep rumen fluid was clarified by centrifugation (Hobson, 1969), and ovine pancreas was autolysed at 4 °C for 3 days as described by Thomas (1963).

Quantities of all ingredients used are given as their final concentrations in media.

Preparation of solidified media. The hoof agar medium described by Parsonson *et al.* (1967) was used. The basal portion contained (g/100 ml water): proteose-peptone, 0.5; Lab-lemco, 0.5; yeast extract, 0.2; agar, 1.5; pH 7.6. The complete medium contained, in addition, 2 g powdered hoof; this was replaced in experimental media by other supplements, most of which were added to the basal medium before autoclaving.

Plates were poured in 9 cm glass Petri dishes from 20 ml volumes of sterile, molten medium, and after setting were placed, opened and inverted, in a 37 °C incubator to dry for 15 min; before use they were stored for at least 12 h under hydrogen in anaerobic jars (Baird & Tatlock Ltd, Chadwell Heath, Essex), each fitted with two catalyst sachets.

Preparation of liquid media. The following simplification of classical anaerobic techniques devised and reviewed by Hungate (1969) proved satisfactory for growing *B. nodosus*. Glass bottles (28 ml) containing 10 ml freshly prepared medium were used as culture vessels; they were fitted with perforated metal screw caps having liners of 3 mm thick rubber. The caps were loosely screwed on before sterilization of the medium by autoclaving. After the autoclave chamber had returned to atmospheric pressure, bottles were removed immediately and the caps tightened. A partial vacuum developed within each bottle as it cooled to room temperature, when deoxygenated CO₂ or N₂ was introduced from the gas line through a sterile 22-gauge hypodermic needle plunged through the liner. Sterility of the gas was ensured by attaching a short length of glass tubing plugged with cotton wool to the base of the needle as a filter, both needle and filter having been autoclaved together before attachment to the gas line. Thereafter, the needle was sterilized by flaming. Gas was allowed to flow into the bottle until a slight positive pressure was registered by an in-line manometer when the needle was withdrawn. Inoculum and sterile heat-labile medium constituents were injected into the culture bottles with sterile syringes bearing 26-gauge needles. This technique was suitable for screw-capped bottles up to 1 l in capacity, which could be half to two-thirds filled with medium.

In general, experiments were performed by adding various nutrients anaerobically to a concentrated base and diluting to final volume.

Measurement of growth on solidified media. Growth was assessed by the gross appearance of colonies and was described as good, fair, poor or nil.

Measurement of growth in liquid media. Growth was measured with an EEL Nephelometer (EEL International Ltd, Bayswater, Victoria, Australia) using an OR2 filter. The instrument was set to read 100 against a supplied standard and a sample of uninoculated medium served as the blank. If necessary, cultures were diluted with sterile medium so that turbidities fell within the scale range. EEL readings were calibrated as equivalent direct bacterial counts as measured in a Petroff-Hausser chamber (Arthur H. Thomas Co, Philadelphia, U.S.A.), 50 EEL units being equivalent to 2.75×10^8 bacteria/ml.

Sterilization. Media and heat-stable stock solutions were autoclaved in culture bottles at 121 °C for 15 min, after which the caps were tightened to exclude atmospheric oxygen. Solutions of heat-labile compounds were sterilized by passage through Millipore Swinnex-13 filter units with 0.45 µm porosity membranes.

Independently-sterilized medium ingredients were withdrawn from concentrated stock solutions with sterile syringes after first relieving the vacuum within the container by the injection of sterile, oxygen-free CO₂ or N₂ as described earlier. Required amounts were then injected into the culture bottles of basic medium.

Inoculation and incubation of cultures. Agar plates that had been stored under hydrogen were inoculated in air by streaking and then replaced promptly inside the anaerobic jar

which was evacuated and filled with a mixture of hydrogen and carbon dioxide (9:1, v/v) twice in succession. Incubation was at 37 °C for up to 5 days.

Freshly-revived lyophilized cultures were grown on agar for 48 h to provide inocula for experimental liquid media. Growth was harvested in freshly autoclaved 0.15 M-NaCl, washed twice and finally resuspended in saline (0.85 % NaCl) at a density of approximately 10^8 bacteria/ml. Triplicate culture bottles, each containing 10 ml test medium, were then inoculated with 0.1 ml volumes of this suspension.

When a reproducible liquid maintenance medium had been developed, bottle cultures were initiated with growth from agar plates and thereafter propagated by inoculating fresh medium with 0.01 vol. culture in the exponential phase of growth. The first or second of such subcultures was used to prepare diluted inocula for the critical assessment of the essential nature of various nutrients and their concentrations supporting optimal growth. Bacteria were concentrated by centrifugation, washed twice and resuspended in autoclaved 0.15 M-NaCl at a density of 10^7 organisms/ml; 0.05 ml of this suspension was inoculated into each 10 ml volume of assay medium.

All bottle cultures were incubated at 37 °C in a water bath and subsequently could be stored at 4 °C for up to 5 days before subculturing. Each growth experiment was performed with at least three *B. nodosus* strains.

Determination of CO₂ requirement. Confirmation of a requirement for CO₂ was sought by comparing the growth of liquid cultures in the presence of NaHCO₃, Na₂CO₃ or CO₂ gas with that under CO₂-free conditions. Preparation of a CO₂-free medium was accomplished by adjusting the pH of a solution of all ingredients of the final liquid medium (Table 1) except Na₂CO₃ to 5.5 with 3 M-HCl before dispensing. The bottles of medium were then autoclaved for 15 min at 121 °C, the caps tightened and, after cooling, gassed with nitrogen. Finally, the pH was readjusted to 6.8 with freshly prepared 2.5 M-NaOH.

RESULTS

Preliminary experiments with solidified media

Strains of *B. nodosus* originally isolated on hoof agar survived weekly transfer on the same medium for several months. Cultures incubated under H₂+CO₂ for 4 to 5 days resulted in fair growth as flat, colourless colonies and up to 3 mm in diameter, with irregular margins; as first noted by Beveridge (1941), they produced a characteristic central pitting of the agar surface (Fig. 1). No growth occurred on the basal medium alone, or on the complete medium when plates were incubated in air, in 100 % H₂, or in 100 % CO₂.

In contrast to hoof powder, equivalent amounts of neutralized acid or alkaline hydrolysates of hoof supported poor or sporadic growth. This situation was somewhat improved when the medium contained a diffusate of a hoof powder suspension added before autoclaving. Streaked cultures also grew on the surface of 5 × 7 cm strips of dialysis membrane (Visking) that had been separately sterilized and placed directly upon plates of hoof agar before inoculation.

Further experiments showed that sodium sulphide, sodium thioglycollate or cysteine-HCl was not an effective substitute for hoof powder, but that reduced glutathione (1 mM), autoclaved with the basal medium, gave fair but consistent growth. However, growth in the form of more elevated, opaque colonies was substantially increased when the medium was supplemented with trypsin-digested casein (Trypticase, 1.5 %, w/v). Acid-hydrolysed casein (Casamino acids, 0.5 %, w/v), on the other hand, was inactive.

Growth in liquid media

In preliminary attempts to simulate the Trypticase plating medium with a liquid version dispensed under CO₂ or CO₂+N₂ mixtures, only faint growth of cultures was obtained after incubation for 3 to 4 days. When supplementary carbohydrates, nitrogen sources, growth factors and metals were screened for growth-promoting activity it was found that either Casamino acids (5 mg/ml) or a mixture of 20 amino acids (0.2 to 1.0 mg/ml) enhanced growth of cultures incubated for 2 to 3 days. Eventually, this stimulatory effect was traced to arginine and serine, and when the optimal requirement for both amino acids in the Trypticase medium had been established, significant increases in the growth rate and final densities of the bacterial cultures resulted.

Further growth increments were then sought from other supplements to the Trypticase-arginine-serine combination in the presence of sodium thioglycollate and 100 % CO₂. They included haemin (5 µg/ml), menadione (0.2 µg/ml), volatile fatty acids, Krebs-cycle acids, hexoses (all at 0.06 mmol/ml), Tween 80 (Atlas Chemical Industries, Inc., U.S.A.; 1 mg/ml), and Ca²⁺ and Mg²⁺ (0.01 to 3.0 mg/ml). Additional materials reputedly stimulatory for anaerobic bacteria were ox bile (Beerens & Castel, 1960), liver digest and yeast extract mixture (Khairat, 1966), and isobutyric acid and bicarbonate mixture (Hardy & Munro, 1966). Of these, only magnesium (0.2 mg/ml), as either the sulphate or chloride salt, increased growth.

Final liquid medium

The final composition of the Trypticase-arginine-serine (TAS) medium is shown in Table 1. Omission of any of the ingredients prevented or impaired growth, and the concentration of each which promoted the highest bacterial densities of three *B. nodosus* strains was assayed independently. Growth yields in TAS medium were not improved by including aqueous extracts of hoof powder, of trypsin or of *B. nodosus*, or by adding sterile filtrates of liver digest (3 to 10 mg/ml), ovine pancreas autodigest, bovine blood serum or rumen fluid (5 to 10 %, v/v). However, final growth of four strains was slightly increased (10 to 20 %) in the presence of 0.01 M-lysine-HCl.

All *B. nodosus* isolates available grew in TAS medium, and of 20 strains examined in detail, all survived ten or more consecutive transfers using as inoculum 0.01 to 0.02 volumes of exponential phase cultures. Even so, there were considerable differences between strains, including recent isolates, in both the time taken to reach maximum population densities after inoculation (range, 16 to 36 h) and their maximum growth yields (range, 1.75×10^8 to 8.0×10^8 organisms/ml). In general, bacteria grown in liquid media were shorter and wider than those from corresponding agar cultures, although this tendency also varied between strains. Extended incubation of stationary phase cultures was accompanied by a slow decline in culture turbidities, but the extensive lysis of bacteria at the end of the exponential phase recorded by Walker *et al.* (1973) was not observed.

No culture grown in TAS liquid medium achieved a bacterial density as high as 2×10^9 organisms/ml, as was reported for two *B. nodosus* strains studied by Walker *et al.* (1973). The atmospheric and temperature requirements for cultivating these organisms were not disclosed, but the maximum growth of Australian and New Zealand isolates incubated at 37 °C under 100 % CO₂ in a similar medium (3 % Tryptone + 0.5 % yeast extract) did not exceed 1.5×10^8 organisms/ml. Attempts to select mutant organisms with enhanced growth in this medium by prolonging incubation of heavily seeded cultures and sub-culturing at intervals proved unsuccessful.

Fig. 1. Colonies of *B. nodosus* grown on hoof agar (Thomas, 1958) for 5 days, showing central etching of the agar surface.

Fig. 2. Growth response of (a) *B. nodosus* strain 4 (McMaster Laboratory strain 131) and (b) strain 10 as a function of changes in the concentrations of arginine and serine in the medium described in Table 1. DL-Serine concentrations: □, nil; △, 15 mm; ○, 30 mm.

Table 1. *Final composition of liquid TAS medium*

A solution in glass-distilled water of all ingredients except Na_2CO_3 was adjusted to pH 7.0 with 2.5 M-NaOH and dispensed in screw-capped culture bottles to about 2/3 capacity. After autoclaving, the bottles were sealed and injected when cool with sterile Na_2CO_3 solution and CO_2 .

Component	Amount/l
Trypticase	15.0 g
Lab-lemco	5.0 g
Proteose-peptone	5.0 g
Yeast extract	2.0 g
L-Arginine-HCl	5.0 g
DL-Serine	1.5 g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	2.0 g
Thioglycollic acid, 95 % (v/v)	0.63 ml*
Na_2CO_3 , 20 % (w/v)	10 ml†

pH before autoclaving 7.0

* Reagent stored in screw-capped bottles at 4 °C under oxygen-free nitrogen and withdrawn by syringe

† Sterilized separately by autoclaving.

Fig. 3. Typical morphology of colonies from a culture of *B. nodosus*, incubated on TAS agar for 4 days, showing central cone arising from an etched depression in the agar and thin surrounding growth.

Fig. 4. Swarming, non-etching colony from a culture of *B. nodosus* incubated on TAS agar for 4 days, showing thin, spreading growth and lack of central features.

Specific growth requirements

Arginine and serine. Growth of two strains of *B. nodosus* in liquid medium, as a function of the concentrations of arginine and serine, is illustrated in Fig. 2. Glutamate, glutamine, citrulline or ornithine could not replace arginine in promoting growth; 0.02 to 0.05 M-L-arginine-HCl was optimal for all strains. DL-Serine (approx. 15 mM) was only slightly less effective in stimulating growth than similar concentrations of L-serine.

Protein hydrolysate. Despite the presence of proteose-peptone and meat extract in TAS medium, additional enzyme-hydrolysed protein in the form of Trypticase was necessary to stimulate prompt growth of cultures from small inocula (Table 2). Two different

Table 2. *Comparison of Trypticase with other protein hydrolysates in stimulating growth of three strains of B. nodosus*

Protein preparation*	Source	Hydrolysis	Growth of <i>B. nodosus</i> † (EEL units)					
			Strain 29		Strain 37		Strain 80	
			10 h	40 h	20 h	40 h	20 h	40 h
None	—	—	0	6	0	49	4	40
Trypticase	Casein	Pancreatic	14	67	29	68	74	65
Tryptone	Casein	Pancreatic	0	49	0	69	6	48
Casitone	Casein	Pancreatic	0	28	2	64	5	50
Lactalsate	Lactalbumin	Pancreatic	0	44	2	44	5	35
Myosate	Cardiac	Pancreatic	0	0	6	64	6	46
Gelysate	Gelatin	Pancreatic	0	4	0	55	4	15
Thiotone	Animal	Peptic	0	9	6	56	13	53
Phytone	Soya bean	Papaic	0	2	3	56	3	42
Liver digest	Ox liver	Papaic	0	3	0	47	5	20
Trypsin	Pancreas	Autolytic	0	0	0	8	4	8
Casamino acids	Casein	HCl	0	10	0	12	3	53

* Added to the basal medium to give a final concentration of 10 mg/ml, except for liver digest and Casamino acids, 5 mg/ml, and trypsin autolysate (Thomas, 1963), 0.1 ml/ml. The basal medium was similar to that described in Table 1, with Trypticase omitted.

† Mean of triplicate cultures incubated for 20 and 40 h.

commercial pancreatic digests of casein, Casitone and Tryptone, were not as uniformly effective as Trypticase for all strains, while other forms of hydrolysed protein gave delayed and inconsistent growth. Although the optimum concentrations of Trypticase supporting good rapid growth varied between strains, a level of 15 mg/ml was eventually adopted for the final medium.

Reducing agents. As shown in Table 3, reducing agents – not necessarily of a thiol type – were essential for the initiation of growth of liquid cultures. Thioglycollate proved the most effective reagent when included in the medium before autoclaving. More reproducible results were obtained by using thioglycollic acid solution than with different batches of sodium thioglycollate.

Carbon dioxide. No growth occurred when the CO₂-free medium was inoculated with a dilute suspension of washed bacteria. This was unlikely to have been due to a lack of buffering capacity, because a similar result was obtained when the medium was buffered to pH 6.8 with 0.025 M-K₂HPO₄-KH₂PO₄. Growth did occur, however, when this medium was supplemented with NaHCO₃, the densest cultures being produced in the presence of Na₂CO₃ and a gas phase of 50 to 100 % CO₂ (Table 4).

Temperature and pH. The optimum growth temperature determined for bottle cultures was 37 to 39 °C. After equilibration with CO₂ and Na₂CO₃, the pH of sterile TAS medium in culture bottles was 6.8 to 6.9. The pH of cultures at the end of the exponential phase ranged from 7.1 to 7.3.

Solidified media

The composition of maintenance and isolation plating media, complementing that of TAS liquid medium, is shown in Table 5. Since no stimulatory effect by Mg²⁺ could be demonstrated on the growth of agar cultures, it was omitted from both formulations.

Maintenance medium. Good growth of cultures was obtained on this medium after incubation for 3 to 4 days. A typical individual colony consisted of a central, raised cone

Table 3. *Effects of different reducing agents on growth of three strains of B. nodosus*

Compound†	Concentration (mM)	Maximum increase in turbidity* (EEL units) after incubation		
		Strain 10 (18 h incubation)	Strain 74 (40 h incubation)	Strain 80 (26 h incubation)
None	—	1	0	2
Sodium thioglycollate‡	10	76	50	49
Sodium thioglycollate	10	42	16	26
Ascorbic acid	15	65	31	49
Cysteine-HCl	2.5	50	59	43
Sodium formaldehyde sulphonylate	2.0	61	28	56
Glutathione (reduced)	3.0	54	38	23
Dithiothreitol	1.0	4	4	0
Dithiothreitol + cysteine-HCl	1.0 + 2.5	0	0	0

* Mean for 3 replicate cultures.

† Added to the autoclaved basal medium as a filter-sterilized solution. The basal medium was similar to that described in Table 1, with thioglycollic acid omitted.

‡ Included in medium before autoclaving.

Table 4. *Growth response of three strains of B. nodosus to increasing concentrations of CO₂*

NaHCO ₃ concn (mg/ml CO ₂ -free basal medium)	Gas phase	Maximum increase in turbidity* (EEL units) after incubation		
		Strain 10 (18 h incubation)	Strain 29 (20 h incubation)	Strain 80 (26 h incubation)
0	N ₂	2	6	0
0.5	N ₂	4	5	4
1.0	N ₂	5	7	5
2.0	N ₂	38	31	8
5.0	N ₂	38	21	19
10.0	N ₂	15	8	30
0	CO ₂ + N ₂ , 1:9	48	3	7
0	CO ₂ + N ₂ , 1:1	59	57	18
0	CO ₂	66	56	29
Na ₂ CO ₃ , 2 mg/ml	CO ₂	82	68	67

* Mean of 3 replicate cultures.

Table 5. *Composition of TAS agar maintenance and isolation media**

Component	Maintenance medium (g/l)	Isolation medium† (g/l)
Trypticase	15.0	15.0
Lab-lemco	5.0	5.0
Proteose-peptone	5.0	5.0
Yeast extract	2.0	2.0
L-Arginine-HCl	5.0	5.0
DL-Serine	1.5	1.5
Washed, packed bovine erythrocytes	—	(5.0 ml/l)
Agar	15.0	22.0
pH before autoclaving	7.8	8.0

* Cultures incubated under 90 % (v/v) H₂ + 10 % (v/v) CO₂.† A Petri dish containing 40 g granular CaCl₂ was placed inside the anaerobic jar together with inoculated plates.

Fig. 5. Filamentous cells from poorly growing *B. nodosus* cultures on agar plates incubated under suboptimally anaerobic conditions (Carbol-fuchsin stain).

Fig. 6. Typical *B. nodosus* colonies (arrows) growing on TAS isolation agar inoculated directly with necrotic material from an ovine foot-rot lesion.

(approx. 1 mm diam.) lying within, and more or less concealing, a circular pit in the agar; beyond this a thin veil of growth extended in the form of a halo to an overall diameter of up to 5 mm (Fig. 3). Unfixed bacteria from these cultures, examined by phase contrast, were stout, slightly curved rods with rounded ends; their modal dimensions were $6.4 \times 1.0 \mu\text{m}$.

Occasionally, a swarming type of growth appeared on plates in the form of flat colonies up to 22 mm in diameter; these usually lacked central features and did not etch the agar surface (Fig. 4). Many strains, particularly after repeated subculturing, displayed this swarming phenomenon, which appeared to be associated with a higher than usual degree of hydration of the agar surface since subsequent subcultures inoculated on plates that had been dried for longer periods yielded colonies of typical appearance.

Excessive drying of agar plates at elevated temperatures immediately before inoculation led to oxidative changes that restricted growth in a similar manner to agar cultures incubated in anaerobic jars containing used catalysts whose effectiveness had declined. Growth under these conditions was limited to clusters of etching, pin-point sized colonies which mainly consisted of atypical, filamentous bacilli up to $80 \mu\text{m}$ long (Fig. 5), suggesting that processes associated with division rather than biosynthesis might be especially dependent upon chemically-reduced medium components. Subculture of this aberrant growth under more favourable conditions yielded *B. nodosus* colonies and organisms of typical morphology.

Isolation medium. The isolation of *B. nodosus* colonies from cultures of infected hoof tissue is greatly impeded by numerous organisms from faecal and environmental sources. Dilution of samples of necrotic material in 0.25 M-sucrose before culturing was advocated by Merritt (1960) as a means of controlling growth of these contaminants, particularly motile forms which spread rapidly across the surface of the medium. A similar result was achieved by increasing the concentration of agar and the pH of the TAS maintenance medium before autoclaving, and by including a dessicant with inoculated plates in the anaerobic jar. These modifications did not alter the appearance of typical *B. nodosus* colonies and since these are more readily distinguished when plates are examined under reflected light (Fig. 6) a suspension of bovine erythrocytes was also included in the medium to increase its opacity. As undiluted samples could be inoculated directly on to this medium, a greater number of *B. nodosus* isolates could be recovered from each primary isolation plate.

DISCUSSION

In view of the tissue specificity of *B. nodosus* infection *in vivo* (Thomas, 1962; Egerton *et al.* 1969), the nutritional activity of hoof powder in artificial media devised by Thomas (1958) was a finding of considerable ecological interest. Dialysable peptides, which may be produced from horn by dry grinding (Cohen, 1944), were implicated in the present work as a source of this activity which was also reproduced by enzyme-hydrolysed but not by acid-hydrolysed casein. Further work is required to establish the nature and specificity of the factors concerned.

As keratins in general have a higher arginine content than most proteins (Ward & Lundgren, 1954), hoof powder might also have provided Thomas's media with significant amounts of a nutrient that proved essential for all strains of *B. nodosus* examined. Although arginine cannot be replaced in culture media by citrulline or ornithine, it is rapidly degraded by washed cell suspensions to citrulline, ornithine and ammonia, ornithine being further converted to putrescine and CO_2 (Skerman, unpublished data). This evidence, together with the relatively large amount of arginine needed for growth and the apparently low

carbohydrate demand of the organism, suggests that arginine might constitute a primary energy source for *B. nodosus* and might be catabolized by constitutive arginine deaminase and ornithine transcarbamylase enzymes, with ATP being ultimately generated from carbamyl phosphate.

The freedom of anaerobic TAS media from particulate and heat-precipitable constituents facilitated their preparation by standard autoclaving procedures and the harvesting of bacteria from liquid cultures. The culture techniques described here have been adapted for growing overnight batch cultures in sealed laboratory fermenters of up to 20 l capacity, with provision for the anaerobic addition of CO₂, Na₂CO₃ and inoculum (0.01 volumes). In previous experiments (Skerman & Cairney, 1972) prompt growth of suitable strains of *B. nodosus* was thus achieved in cultures in which factors associated with virulence and immunogenicity were conserved.

Quantitative growth differences that have been noted among isolates may reflect physiological disparities which have yet to be determined. Detailed taxonomic analysis will also be required to determine whether these are associated with other distinctions between strains, in particular, their virulence (Egerton & Parsonson, 1969) and their antigenic properties (Egerton, 1974).

The technical assistance of Mr G. Boucher, Miss Elaine O'Connell and Miss Sylvia Bauer is gratefully acknowledged. I also thank Mr A. Barkus for the photography, Professor J. R. Egerton for Australian isolates of *B. nodosus* and Dr W. A. Te Punga for helpful comments.

REFERENCES

- BEERENS, H. & CASTEL, M. M. (1960). Action de la bile sur la croissance de certaines bactéries anaérobies à gram-négatif. *Annales de l'Institut Pasteur* **99**, 454-456.
- BEVERIDGE, W. I. B. (1941). *Foot-rot in Sheep: a Transmissible Disease Due to Infection with Fusiformis nodosus (n.sp.)*. Commonwealth of Australia Council for Scientific and Industrial Research Bulletin No. 140, pp. 1-64.
- BEVERIDGE, W. I. B. (1956). Foot-rot of sheep. A modern approach to an old problem. *Veterinary Record* **68**, 963-965.
- BRENNER, M., NIEDERWIESER, A. & PATAKI, G. (1965). Amino acids and derivatives. In *Thin-layer Chromatography. A Laboratory Handbook*, pp. 381-432. Edited by E. Stahl. New York and London: Academic Press.
- COHEN, H. R. (1944). The effect of dry grinding on the properties of proteins. V. Keratins. *Archives of Biochemistry* **4**, 145-150.
- EGERTON, J. R. (1974). Significance of *Fusiformis nodosus* serotypes in resistance of vaccinated sheep to experimental foot-rot. *Australian Veterinary Journal* **50**, 59-62.
- EGERTON, J. R. & BURRELL, D. H. (1970). Prophylactic and therapeutic vaccination against ovine foot-rot. *Australian Veterinary Journal* **46**, 517-522.
- EGERTON, J. R. & PARSONSON, I. M. (1969). Benign foot-rot - a specific interdigital dermatitis of sheep associated with infection by less proteolytic strains of *Fusiformis nodosus*. *Australian Veterinary Journal* **45**, 345-349.
- EGERTON, J. R., ROBERTS, D. S. & PARSONSON, I. M. (1969). The aetiology and pathogenesis of ovine foot-rot. I. A histological study of the bacterial invasion. *Journal of Comparative Pathology* **79**, 207-219.
- HARDY, P. H. & MUNRO, C. O. (1966). Nutritional requirements of anaerobic spirochaetes. I. Demonstration of isobutyrate and bicarbonate as growth factors for a strain of *Treponema microdentium*. *Journal of Bacteriology* **91**, 27-32.
- HOBSON, P. N. (1969). Rumen bacteria. In *Methods in Microbiology*, vol. 3B, pp. 133-149. Edited by J. R. Norris and D. W. Ribbons. London and New York: Academic Press.

- HUNGATE, R. E. (1969). A roll tube method for cultivation of strict anaerobes. In *Methods in Microbiology*, vol. 3B, pp. 117-132. Edited by J. R. Norris and D. W. Ribbons. London and New York: Academic Press.
- KHAIRAT, O. (1966). An efficient general-purpose culture medium for aerobes and anaerobes. *Canadian Journal of Microbiology* **12**, 323-331.
- MACKENZIE, J. (1956). Foot-rot in sheep. *Veterinary Record* **68**, 1060.
- MARSH, H. & CLAUS, K. D. (1970). The diagnosis of foot-rot in sheep. *Cornell Veterinarian* **60**, 309-317.
- MERRITT, G. C. (1960). The isolation of *Fusiformis nodosus*. *Australian Veterinary Journal* **36**, 388.
- MOORE, W. E. C. (1966). Techniques for routine culture of fastidious anaerobes. *International Journal of Systematic Bacteriology* **16**, 173-190.
- PARSONSON, I. M., EGERTON, J. R. & ROBERTS, D. S. (1967). Ovine interdigital dermatitis. *Journal of Comparative Pathology* **77**, 309-313.
- ROBERTS, D. S. (1967). The pathogenic synergy of *Fusiformis necrophorus* and *Corynebacterium pyogenes*. I. Influence of the leucocidal exotoxin of *F. necrophorus*. *British Journal of Experimental Pathology* **48**, 665-673.
- SKERMAN, T. M. (1972). A nutrient culture medium for *Bacteroides nodosus* and the preparation of a vaccine against foot-rot in ruminants. New Zealand Inventions Development Authority Patent Application No. 160727. *New Zealand Patent Office Journal* **61**, 899.
- SKERMAN, T. M. & CAIRNEY, I. M. (1972). Experimental observations on prophylactic and therapeutic vaccination against foot-rot in sheep. *New Zealand Veterinary Journal* **20**, 205-211.
- THOMAS, J. H. (1958). A simple medium for the isolation and cultivation of *Fusiformis nodosus*. *Australian Veterinary Journal* **34**, 411.
- THOMAS, J. H. (1962). The bacteriology and histopathology of foot-rot in sheep. *Australian Journal of Agricultural Research* **13**, 725-733.
- THOMAS, J. H. (1963). A liquid medium for the growth of *Fusiformis nodosus*. *Australian Veterinary Journal* **39**, 434-437.
- WALKER, P. D., SHORT, J., THOMPSON, R. O. & ROBERTS, D. S. (1973). The fine structure of *Fusiformis nodosus* with special reference to the location of antigens associated with immunogenicity. *Journal of General Microbiology* **77**, 351-361.
- WARD, W. H. & LUNDGREN, H. P. (1954). The formation, composition, and properties of the keratins. In *Advances in Protein Chemistry*, vol. 9, pp. 243-297. Edited by M. L. Anson, K. Bailey and J. T. Edsall. New York: Academic Press.