SHORT ARTICLES

THE EFFECT OF DIFFERENT ENVIRONMENTAL CONDITIONS ON SOME CHARACTERS OF *HAEMOPHILUS PARAPHROPHILUS*

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THERE is a confusing difference of opinion in the literature about the part played by increased CO₂ tension in the optimal growth of a number of bacteria. It has been accepted for many years that incubation in air with added CO₂ is essential for growth, or promotes better growth, of certain species (Wilson, 1931; McLeod *et al.*, 1934). The increased tension of CO₂ is needed chiefly for first isolation of some species, but also for maintenance culture of others. The CO₂ appears to have different roles in different organisms, e.g., (1) it functions as an essential growth factor for mutants of *Escherichia coli* (Charles and Roberts, 1968), (2) it is required for the normal morphological development of pneumococci (Austrian and Collins, 1966), and (3) it increases the percentage of X-independent variants of some strains of *Haemophilus aphrophilus* (Boyce, Frazer and Zinnemann, 1969). Zinnemann *et al.* (1968) described *Haemophilus paraphrophilus* as requiring increased CO₂ tension for optimal growth, and sometimes also for primary isolation. They stated that the organism formed typical Gram-negative bacilli and gave good growth with moist colonies only when incubated with added CO₂. In the absence of NaCl, V-dependent growth took place only in the presence of increased CO₂.

Our attention was drawn by Dr D. E. Nicholson to the fact that a sealed jar, without any gaseous addition, could be used to promote good growth of primary cultures of *Neisseria gonorrhoeae*. This information prompted us to investigate further the effect of different environments, including increased CO₂ tension, on some characters of *H. paraphrophilus*.

MATERIALS AND METHODS

Strains

These were three strains of *Haemophilus paraphrophilus* described by Zinnemann *et al.*, now in the National Collection of Type Cultures under the reference numbers 10556, 10557 and 10558, and two other strains, Jordan and Plummer, obtained from Dr K. B. Rogers.

All strains were identified as being consistently V-dependent when incubated in air with 5–10 per cent. (v/v) CO₂ in tests by the method of Zinnemann *et al.* A standard inoculum of the organism from a 24-hr growth on "chocolate" (heated blood) agar was spread on to different media containing V-factor. Growth in air with added CO₂ and absence of growth in air without added CO₂ differentiated *H. paraphrophilus* from other V-dependent species of *Haemophilus*.

Culture media

Yeastrel agar (YA). This was 3 per cent. autoclaved Yeastrel (yeast extract) agar without added NaCl (Zinnemann, 1960). *Chocolate agar* was 10 per cent. (v/v) heated horse blood in nutrient agar. It was used for maintenance cultures and for demonstrating the microscopic and naked-eye appearances of cultures grown under different experimental conditions. *Proteose peptone agar (PPA)*. This was 2 per cent. proteose peptone agar No. 3 (Difco) plus 0.6 per cent. NaCl (Zinnemann *et al.*). It was used as a V-deficient medium in tests for V-dependence, as was YA.

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Growth factors. V-factor was supplied in two forms: (1) by inoculation of the Oxford (Heatley) strain of *Staphylococcus aureus* as a "feeder" organism from a 24-48-hr growth on nutrient agar, and (2) by addition of 1 μ g of beta-diphosphopyridine nucleotide (DPN) (Sigma Grade III) per ml.

Culture methods

Inoculation. The bacteria to be inoculated were swept up with a loop from an 18-24-hr growth on a chocolate agar plate and suspended in quarter-strength Ringer's solution to a concentration of 1×10^8 viable bacteria per ml. The inoculum for plates was a 3-mm diam. loopful of this suspension.

Five different media were used: YA, YA+DPN, PPA, PPA+DPN, and chocolate agar. Quadrants of each plate were spread with suspensions of four of the *H. paraphrophilus* strains under investigation. A light streak of feeder staphylococcus was then inoculated on YA and PPA plates.

TABLE

Macroscopical and microscopical appearances of cultures of Haemophilus paraphrophilus grown on plates of "chocolate" agar incubated under different conditions of atmospheric humidity and CO₂ tension

Conditions of incubation	Macroscopical appearance	Microscopical appearance
On shelf (S)	Rough, dry	Involution forms*
In closed jar (C)	Smooth, moist	Involution forms
On sealed plates (S/Se)	Smooth, moist	Involution forms
In jar with excess moisture (C/H ₂ O)	Smooth, moist	Involution forms
In jar with 5-10 per cent. CO ₂ (C/CO ₂)	Smooth, moist	Coccobacilli
In jar with 5-10 per cent. CO ₂ and dried CaCl ₂ (C/CO ₂ /CaCl ₂)	Rough, dry	Involution forms

* Filaments and chains.

Incubation. The five media were incubated at 37°C under each of the following sets of conditions: (1) on the shelf of the incubator under conventional conditions (S), (2) in a closed jar on the incubator shelf (the jar was first emptied of possible traces of CO₂ from previous experiments by standing it upside down over the edge of the bench for several hours) (C), (3) on plates individually sealed with strips of plasticine and placed on the incubator shelf (S/Se), (4) in a closed jar containing a large boiling tube with water and a large wad of cotton wool saturated in water (C/H₂O), (5) in a closed jar containing 5-10 per cent. added CO₂, produced by adding 10-15 ml concentrated H₂SO₄ to 5 or 6 small marble chips in a boiling tube (C/CO₂), and (6) in a closed jar with 5-10 per cent. added CO₂, but with the addition of a dish of freshly fused CaCl₂ (C/CO₂/CaCl₂). The plates, which had been thoroughly dried before use, were kept open with plasticine pellets on the inside edge of the lid. This was done in an attempt to remove all free moisture from the jar and plates. However, when the plates were returned to the bench after incubation, fine droplets soon developed round the inside edges showing that there was still free moisture available.

After they had been incubated for 24 and 48 hr, they were examined for microscopical appearance, colonial growth, and V-dependent growth.

RESULTS

Microscopical appearance. Typical Gram-negative coccobacillary forms were found only in the cultures incubated in the closed jar with 5-10 per cent. CO₂ (C/CO₂). Many involution forms and filaments were seen in cultures grown under the other five sets of experimental conditions.

Colonial growth. Abundant smooth moist growth was observed on sealed plates and in all closed jars except the one that contained CaCl_2 . Dry "bread-crumbs" growth was obtained in this jar ($\text{C}/\text{CO}_2/\text{CaCl}_2$) and also on the plates incubated on the shelf (S). The macroscopical and microscopical appearances of the different cultures are listed in the table.

V-dependence. With the "feeder" staphylococcus as the source of V-factor, satellitism was obtained around the streak of growth of *H. paraphrophilus* on YA under all conditions of incubation except incubation on the shelf (S). There was satellite growth on the control PPA plates under all conditions of incubation. With DPN as the source of V-factor, growth was absent on YA incubated on the shelf (S) and inconsistent on YA under all other conditions of incubation. There was growth on the control PPA plates under all conditions except on the shelf (S), where the results were again inconsistent.

DISCUSSION

Typical small coccobacilli, and abundant, smooth, moist growth of *Haemophilus paraphrophilus* were obtained only under conventional conditions in a CO_2 jar, i.e., with an increased tension of CO_2 and an increased content of moisture in the atmosphere. The experiments showed that V-dependent growth could take place on basic media plus V-factor when either (1) the moisture content of the atmosphere was increased (or the normal degree of evaporation prevented) or (2) an increased concentration of CO_2 was provided. The PPA plates with added V-factor were used as controls because they contained 0.6 per cent. NaCl and because previous work had shown that addition of 0.6 per cent. NaCl to media enabled V-dependent growth to take place under normal atmospheric conditions (Zinnemann *et al.*, 1968).

The observations on YA were consistent only when V-factor was supplied by the "feeder" staphylococcus, not when it was supplied as DPN. That DPN was not inactive as a source of V-factor was shown by the observation that growth took place on PPA plus DPN under all conditions of incubation. Moreover, when a ten-fold greater concentration of DPN was added to YA, growth still did not develop consistently. It is unlikely, therefore, that the concentration of $1 \mu\text{g}$ of V-factor per ml, which is generally accepted as an adequate supplement, was subliminal for growth of *H. paraphrophilus* in these experiments. The difference in response to the two sources of V-factor is puzzling and open to speculation. It may be that the feeder strain growing on YA is supplying some other factor additional to V, thus enabling the test strain to grow in the presence of increased CO_2 tension, or sufficient moisture, but without NaCl. Alternatively, YA may contain a factor that inhibits the growth of *H. paraphrophilus* in the absence of substances diffusing from the feeder organism, or in the absence of NaCl.

SUMMARY

V-dependent growth of *Haemophilus paraphrophilus*, which Zinnemann *et al.* (1968) found to take place only when cultures were incubated in air with 5–10 per cent. added CO_2 , or on a medium containing 0.6 per cent. NaCl, also takes place in the absence of either of these conditions if the cultures are incubated in a closed, moist atmosphere, as in a sealed plate or a sealed jar.

The findings here described do not provide arguments against recognition of the species *Haemophilus paraphrophilus*, since under conditions of normal humidity and air supply the unadapted, recently isolated strains grow sparsely, or not at all. Typical coccobacillary rods are formed only when cultures are grown in an atmosphere with added CO_2 as well as an increased moisture content.

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AUTO-ANTIBODIES IN PATIENTS WITH CHRONIC PULMONARY TUBERCULOSIS

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ASHERSON AND ROSE (1963) found that rabbits infected with coccidia produced antibodies reacting with homologous organ homogenates. Sera from patients with *Schistosoma haematobium* infections may contain auto-antibodies that fix complement in the presence of normal human liver and lung homogenates (Shamma, Thewaini Ali and El-Shawi, 1965) and stain fresh-frozen human lung and liver sections when used in the fluorescent antibody technique (Shamma, Thewaini Ali and Rassam (1966).

Thewaini Ali and Oakley (1967) found that some rabbits infected with *Mycobacterium tuberculosis* or *Pasteurella pseudotuberculosis* or immunised with sterilised organ homogenates from infected animals develop auto-antibodies reacting with various homologous and heterologous tissue components. They did not provide evidence that auto-antibodies are produced in long-standing tuberculosis in man; the present paper attempts to supply it.

MATERIALS AND METHODS

Blood

Blood was collected from 80 patients suffering from chronic pulmonary tuberculosis with cavitation, but having no history of syphilis, bejel, schistosomiasis, acquired haemolytic anaemia, collagen diseases or infectious mononucleosis, at the Twaitha Tuberculosis Hospital, and from 20 healthy controls. The serum was separated and stored at -25°C until needed.

Auto-antibody tests

Complement-fixation tests. Sera were tested for complement-fixing antibody against human and rabbit liver, lung and kidney homogenates, before and after absorption with rabbit liver or kidney tissue, as described by Thewaini Ali and Oakley.

Anti-nuclear factor tests. The indirect method of Coons and Kaplan (1950) was used; unfixed smears of human buffy coat were treated with the serum under test for 30 min.; the slides were then washed with phosphate-buffered saline (PBS) at pH 7.2, and then treated for 20 min. with one drop of sheep anti-human-globulin conjugated with fluorescein isothiocyanate (Sylvana) absorbed with charcoal and with rat liver powder. The slides were washed again with PBS, drained, mounted in 90 per cent. phosphate-buffered glycerol pH 7.2 and examined for nuclear fluorescence with an ultraviolet microscope. Appropriate controls for the specificity of the fluorescent staining (Thewaini Ali and Oakley) were always included.

Rose-Waaler test. The modified Rose-Waaler test of Rose *et al.* (1948) was used, and the differential agglutination titre was obtained by dividing the reciprocal of the titre obtained