The Effect of Anaerobiosis and Bile Salts on the Growth and Toxin Production by *Vibrio cholerae*

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SUMMARY

Environmental conditions which might be present in the human intestinal lumen, such as anaerobiosis, a temperature of 37°C and the presence of bile salts, were examined for their effects on the growth and toxin production by *Vibrio cholerae* strains 569B and B1307 in Syncase and in peptone water media. Using aerobic conditions at 30°C, which are commonly used for enterotoxin production, toxin (5 µg ml⁻¹) and pleomorphic cells were detected during the exponential phase of the growth cycle. When the incubation temperature was raised to 37°C, no toxin (<0.1 µg ml⁻¹) and no pleomorphic forms were found. In cultures incubated anaerobically at 30 or 37°C, the organisms grew poorly, forming pleomorphic cells which lysed after the cultures reached a maximum turbidity at 640 nm of 1.45 at 12 h. Toxin (2.5 µg ml⁻¹) was present at 12, 24 and 48 h. When 0.1 % sodium deoxycholate was incorporated into the culture medium, growth was inhibited under aerobic conditions at 30 and 37°C. At 30°C under aerobic conditions and at 37°C under anaerobic conditions, the toxin yield was not significantly affected by the presence of sodium deoxycholate; but at 37°C under aerobic conditions, sodium deoxycholate caused an increase in the toxin yield (5 µg ml⁻¹) due to the release of cell-bound toxin.

INTRODUCTION

During the disease cholera, large numbers of *Vibrio cholerae* are present in the small gut (Gorbach *et al.*, 1970). Freter (1969) showed that only those organisms adhering to the intestinal epithelial cells are necessary to produce the disease. At this site, the degree of oxygenation is probably less than that in the type of well-aerated culture vessel generally used in studies on toxin production (Burrows & Kaur, 1974). Although *V. cholerae* grows best *in vitro* under aerobic conditions, von Hirsch (1926) and Banerjee (1939) have demonstrated that it can grow anaerobically. Linton, Mitra & Seal (1936) found that the protein breakdown in cultures grown anaerobically was less than that in cultures incubated under aerobic conditions. However, enterotoxin production under anaerobic conditions has not been well characterized.

The vibrios in the intestine are at 37°C which is the optimal temperature for growth under aerobic conditions (Pollitzer, 1959). However, toxin yield at this temperature *in vitro* under aerobic conditions is less than that in cultures incubated at 25 to 30°C (Craig, 1966; Richardson, 1969).

In the small intestine, *V. cholerae* must grow in an environment which contains bile. Vibrios grow in the presence of bile salts – such salts are incorporated into selective media

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used for their isolation (Pollitzer, 1959) – but they are unable to deconjugate bile salts (Norman & Grubb, 1955). Freter (1972) found that rabbit bile enhanced the adsorption of vibrios to the intestinal mucosa. Other investigators (De, Ghosh & Chandra, 1962) were unable to demonstrate toxin production in cultures of *V. cholerae* grown in medium containing 0.5 % bile salts (Difco). This investigation was undertaken to determine the effects of anaerobiosis, a temperature of 37°C and bile salts on the growth and toxin production *in vitro* by *V. cholerae*.

METHODS

Bacterial strains. Freeze-dried cultures of *V. cholerae* strains 569B and B1307 were obtained from Dr John C. Feeley, N.I.H., Bethesda, Maryland, U.S.A. The rehydrated cultures were passaged twice in rabbit intestinal loops and re-isolated on Brain-Heart Infusion agar (Difco). These were lyophilized and stored at 4°C.

Media. Syncase (Finkelstein *et al.*, 1966) and peptone water (Burrows & Kaur, 1974) were used. In preliminary experiments, the pH of these media fell to approximately 6.0 when kept under anaerobic conditions. The media were therefore buffered with 0.025 M-Tris and the pH was adjusted to 8.0. The Tris-buffered media were prepared in large batches and distributed in 150 ml portions to 250 ml Erlenmeyer flasks fitted with cotton plugs. For anaerobic growth, all media were prereduced by keeping them for 18 h in the anaerobic chamber described below. Media containing bile salts were prepared by adding 1 g (2.2 mM) sodium deoxycholate (Fisher Scientific Co., King of Prussia, Pennsylvania, U.S.A.), 1 g (2.5 mM) sodium glycocholate (Calbiochem) or 1 g Brij-38 (Calbiochem) to 1 l Syncase or peptone water. Sodium glycocholate and sodium deoxycholate were both found to be pure when tested by thin-layer chromatography (Hofmann, 1962).

Growth conditions. Aerobic incubation was carried out at 30 or 37°C in a water-bath shaker operated at 180 rev. min⁻¹ over a 5 cm diam. Anaerobiosis was achieved by two methods. A flexible fibre chamber similar to that described by Aranki & Freter (1972) was used for stationary cultures with an atmosphere in the chamber of 5 % CO₂, 88 % N₂ and 7 % H₂. The oxygen content was approximately 5 p.p.m. as determined by an oxygen analyser (Lockwood and McLorie, Horsham, Pennsylvania, U.S.A.). The oxidation-reduction potential was at least -125 mV as indicated by the reduction of indigo carmine. The relative humidity was 45 to 60 % and the temperature was 37°C.

Cultures to be shaken under anaerobic conditions were inoculated into media prereduced in the chamber. The flasks were placed in Gaspak jars (BBL) in the chamber, and then the jars were removed and placed in water-bath shakers at 30 or 37°C.

Inocula. A fresh vial of lyophilized stock culture was used for each experiment. The culture was reconstituted by adding 1 ml of the medium to be used. A loopful of this was inoculated into 5 ml culture medium and incubated under aerobic or anaerobic conditions at 30 or 37°C without shaking. After incubation for 18 h, 0.1 ml of the culture was inoculated into 10 ml fresh medium and incubated under the appropriate conditions for 3 h; 0.1 ml of this culture was used to inoculate 150 ml culture medium and incubated under the same conditions. At the same time, a count of viable cells in the inoculum was made. Preliminary experiments indicated that the cultures were in the exponential phase of growth at this time.

Estimations of growth. Samples were withdrawn and growth was estimated by four methods:

1. Turbidity was measured at 640 nm in a 15 mm path using a Photovolt Lumetron photometer.
2. Viable counts using serial 10-fold dilutions were made in saline. Duplicate drops (Miles

& Misra, 1938) of each dilution were made and those spots on Brain-Heart Infusion agar plates which contained 25 to 30 colonies were counted.

3. Cells separated by centrifuging (5000 g for 15 min) were dried to constant weight.

4. The weight of material precipitated by 5 % (v/v) cold perchloric acid was determined: samples were treated for 30 min at 4 °C, and the precipitates, separated by centrifuging, were washed once with cold perchloric acid and dried to constant weight. Syncase medium itself gave no precipitate. When media containing bile salts were used, 10 ml samples of cultures were dialysed against several changes of 0.85 % saline before adding cold perchloric acid, since the acid precipitated sodium deoxycholate. Although some macromolecules might have been lost during dialysis, due to breakdown by the action of proteolytic and other degradative enzymes, this method gave higher values than method 3. Method 3 did not give consistently reliable results for cultures grown in media containing bile salts and was therefore not used in such cases.

Phase-contrast microscopy. Agar slides were prepared by layering 2 ml Syncase agar (1.5 % agar in Syncase medium) on sterile microscope slides (75 × 25 mm). A drop of culture was spread on the agar surface and viewed immediately using a phase-contrast microscope (Standard Universal Microscope; Carl Zeiss). The relative numbers of the different morphological types were estimated by counting at least six oil immersion fields in which the organisms were uniformly distributed.

Toxin tests. The supernatant fluid from centrifuged samples of cultures grown under various conditions was filtered through a 0.45 µm membrane filter (Millipore) pre-moistened with fresh, sterile culture medium. The filtrates were collected in containers to which EDTA had been added to give a final concentration of 0.005 %, and were immediately cooled to 4 °C (Richardson, Evans & Feeley, 1970). The filtrates obtained from cultures grown in media containing bile salts were dialysed against several changes of 0.85 % saline at 4 °C for 24 to 30 h. No bile salt was detected in the dialysed filtrates when tested by thin-layer chromatography.

The activities of the filtrates were assayed in two ways:

1. The passive haemagglutination inhibition test (PHI) was used to measure the toxin-antigen *in vivo*. The reagents were prepared as described by Finkelstein & Peterson (1970), and the test was performed as described by Callahan, Ryder & Richardson (1971). Each sample was run in duplicate. Using this technique both active toxin and inactive antigen are estimated together.

2. The adult rabbit ileal loop assay was used to estimate active toxin *in vivo*. This assay was performed according to the method of Pierce & Wallace (1972).

Verification of the presence of enterotoxin. Each sample which gave a positive result in the rabbit was tested to prove that enterotoxin was responsible for the intestinal response:

1. Neutralization with antitoxin (Kasai & Burrows, 1966). Horse anticholeraenoid (Finkelstein, 1970; N.I.H. lot no. 19) was used as antitoxin. The filtrates were incubated with an equal volume of a 1:100 dilution of antitoxin prepared in phosphate-buffered saline, pH 7.2, at 37 °C for 30 min. Ligated rabbit intestinal loops were injected with 0.5 ml of this mixture and with similar volumes of appropriate positive and negative control materials. The effect of the antitoxin on the volume/length ratio was determined 14 to 16 h later when the animals were sacrificed.

2. Test for heat lability (Burrows & Kaur, 1974). Samples of the filtrates which elicited a fluid response in the rabbit ileal loop assay were incubated at 56 °C for 30 min and then tested for residual activity in rabbit intestinal loops.

Sodium deoxycholate treatment. Cells from 12 h cultures (100 ml) grown aerobically at

37°C in Syncase medium were washed once in Syncase medium and resuspended in 25 ml Syncase medium containing 0.1% sodium deoxycholate. After incubating (aerobically at 37°C) for 20 min, with gentle agitation every few minutes, the suspension was filtered and collected in vials containing EDTA. The volume was made up to 100 ml using Syncase medium and the samples were assayed for toxin.

Extraction of enterotoxin by EDTA treatment. Organisms from 100 ml of a 12 h culture were extracted with Tris/EDTA as described by Leive & Shovlin (1968), dialysed and assayed for toxin.

Mechanical lysis of organisms. A dense suspension of the organisms obtained by resuspending the centrifuged, washed sediment of 100 ml culture in 10 ml medium was lysed using an X-Press (Biotec). Phase-contrast microscopy showed that approximately 90% lysis occurred. The lysate was reconstituted to 100 ml in Syncase medium and centrifuged. The supernatant fluid was filtered and assayed for toxin.

RESULTS

Growth and toxin production by V. cholerae 569B

In Syncase medium incubated aerobically. Although there were no significant differences in turbidity, viable counts and dry weight of cold-acid-precipitated cultures at 30 and 37°C, the dry weight of centrifuged culture sediment at 48 h was significantly less at 30°C than at 37°C (Fig. 1). During early-exponential growth, pleomorphic cells were detected at 30°C (Fig. 2a) but not at 37°C (Fig. 2b). As reported previously (Richardson, 1969; Callahan *et al.*, 1971), the toxin yield was greater at 30°C than at 37°C.

In Syncase medium incubated anaerobically at 37°C. There were no marked differences in growth and cellular morphology in stationary or shaken cultures incubated at 30 or 37°C. Maximum turbidity was reached after 12 h incubation (Fig. 3) but was less than that observed under aerobic conditions. Thereafter, turbidity, viable counts and dry weight decreased although the dry weight of cold-acid-precipitated cultures increased. Morphologically, vibrio-like forms predominated after 3 h although a few large rounded cells were seen (Fig. 4a). At 12 h, large round cells with vesicles, some of which appeared extruded, were most evident (Fig. 4b). By 33 h much debris and a few short rod-like or round cells were seen. Toxin yields were good at 12 and 33 h when cultures were incubated at 37°C. When cultures were incubated anaerobically at 30°C, culture turbidity and cellular morphology were similar to that obtained at 37°C, but toxin was not detected until after 33 h incubation.

When the experiments were repeated using peptone water or *V. cholerae* B1307, essentially the same results were obtained.

In Syncase medium containing 0.1% sodium deoxycholate. The presence of the bile salt retarded increases in turbidity, viable counts and dry weight of cold-acid-precipitated cultures (Fig. 5) compared with the increases under similar incubation conditions without sodium deoxycholate. Morphologically the most striking features were the presence at 3 and 12 h of at least 50% of cells with low refractive indices (ghosts), few pleomorphic forms and the remainder appearing to be normal. At 33 h, the number of ghost cells had decreased and the cultures consisted of apparently normal vibrios. Toxin was detected in appreciable quantities after incubation for 33 h aerobically at 30°C (Fig. 5a), for 12 h aerobically at 37°C (Fig. 5b) and for 33 h anaerobically at 37°C (Fig. 5c).

Similar results were obtained when *V. cholerae* B1307 or peptone water containing 0.1% sodium deoxycholate were used.

Fig. 1. Growth and toxin production by *V. cholerae* 569B grown aerobically in Syncase medium incubated with shaking at: (a) 30°C; (b) 37°C. Growth was measured as described in Methods. ●, Viable counts [$\log_{10}$ (colony-forming units) ml^{-1}]; ○, turbidity (E_{640}); ■, dry weight of centrifuged culture sediments (mg ml^{-1}); □, dry weight of cold-acid-precipitated cultures (mg ml^{-1}). Culture filtrates were assayed for toxin-antigen ($\mu\text{g ml}^{-1}$) by the passive haemagglutination inhibition test (PHI), and for biologically active toxin (volume/length response; bar) by the rabbit intestinal loop method. The volume/length ratios are the mean of six values obtained in different rabbits. The distribution indicates the standard error of the mean.

Fig. 2. Morphology of *V. cholerae* 569B at 12 h incubated aerobically at: (a) 30°C; (b) 37°C. Arrows indicate large round cells with vesicles. Bar markers represent 2 μm .

Fig. 3. Growth and toxin production by *V. cholerae* 569B in Syncase medium, incubated anaerobically at 37°C without shaking. For symbols, see legend to Fig. 1.

Fig. 4. Morphology of *V. cholerae* 569B grown anaerobically at 37°C without shaking. (a) At 3 h: arrow indicates large round cell. (b) At 12 h: arrow indicates large round cell with vesicle. Bar markers represent 2 μm .

*Comparison of the amount of extracellular toxin with the intracellular
and/or 'bound' toxin*

The increased toxin yield in cultures incubated aerobically at 37°C in the presence of sodium deoxycholate could be due to (i) increased synthesis of toxin, (ii) release of intracellular toxin, as a result of increased membrane permeability or as a result of cell lysis, or

Fig. 5. Growth and toxin production by *V. cholerae* 569B grown in Syncase medium containing 0.1 % sodium deoxycholate incubated: (a) aerobically at 30°C with shaking; (b) aerobically at 37°C with shaking; (c) anaerobically at 37°C without shaking. Three parameters of growth are shown: ●, viable counts ($\log_{10}$ c.f.u. ml^{-1}); ○, turbidity (E_{640}); □, dry weight of cold-acid-precipitated cultures (mg ml^{-1}). Culture filtrates were assayed for toxin-antigen ($\mu\text{g ml}^{-1}$) by the passive haemagglutination inhibition test (PHI), and for biologically active toxin (volume/length response; bar) by the rabbit intestinal loop method. The volume/length ratios are the mean of six values obtained from different rabbits. The distribution indicates the standard error of the mean.

(iii) release of toxin from a 'bound' form. When 0.1 % sodium deoxycholate was added to organisms grown aerobically at 37°C, the toxin yield in 20 min was $5.0 \mu\text{g ml}^{-1}$ (2.3 *V/l* response) whereas the toxin yield was only $0.2 \mu\text{g ml}^{-1}$ (<0.5 *V/l* response) when Syncase medium containing no sodium deoxycholate was added. The free intracellular toxin released by mechanical lysis was only $0.3 \mu\text{g ml}^{-1}$ (<0.5 *V/l* response). When 0.1 % sodium deoxycholate was added to cell lysates prepared using the X-Press, the toxin yield was $2.5 \mu\text{g ml}^{-1}$ (3.0 *V/l* response).

When organisms grown aerobically at 37°C were treated with Tris/EDTA the toxin yield varied from 0.5 to $2.0 \mu\text{g ml}^{-1}$. This toxin-antigen measured by the PHI assay did not elicit a fluid response in the rabbit intestinal loop. Adding Tris/EDTA to crude cholera toxin (freeze-dried culture filtrate; Wyeth Laboratories, Philadelphia, U.S.A.; lot no. 4493G) did not affect the rabbit intestinal fluid response.

When 0.1 % sodium deoxycholate was added to crude cholera toxin, incubated for 1 h at 37°C and tested in rabbit intestinal loops and by the PHI assay, the results obtained were not significantly different from those obtained with toxin alone.

Effect of sodium glycocholate and Brij-38 on the growth and toxin production by V. cholerae 569B

The addition of 0.1 % sodium glycocholate to Syncase medium did not alter growth and toxin production in cultures incubated aerobically at 30°C and anaerobically at 37°C. Under aerobic conditions at 37°C, the turbidity of the culture was similar to that obtained in medium with no sodium glycocholate but the toxin yield was increased to 2.5 µg ml⁻¹ (2.0 V/l response) at 12 h and 2.5 µg ml⁻¹ (2.2 V/l response) at 33 h.

Growth and toxin production in cultures incubated aerobically at 30°C and anaerobically at 37°C in medium containing 0.1 % Brij-38 was similar to that obtained in medium containing sodium deoxycholate. Under aerobic conditions at 37°C the growth was similar to that obtained in medium containing sodium deoxycholate, but the toxin yield was decreased to 0.63 µg ml⁻¹ (1.7 V/l response) at 12 h and 0.63 µg ml⁻¹ (3.9 V/l response) at 33 h.

DISCUSSION

The toxin yield in *V. cholerae* cultures depends on the environmental conditions in which they are grown. The environmental factors studied by other investigators indicate that aerobic incubation at 30°C yields the largest amounts of toxin (Kusama & Craig, 1970; Richardson, 1969). These incubation conditions are not optimal for growth since we found large numbers of pleomorphic cells, similar to those noted by Kennedy & Richardson (1969), in the exponential growth phase of such cultures. Under aerobic conditions, although the turbidity and viable counts at 37°C were similar to those obtained at 30°C, all the organisms had a 'normal' vibrio shape in the exponential growth phase. Therefore, we confirm Pollitzer's finding (1959) that aerobic incubation at 37°C is optimal for growth, but find the toxin yield is then minimal confirming the work of Craig (1966), Richardson (1969) and Gallut & Jude (1955). In cultures incubated anaerobically at 30 or 37°C, the cellular yield was lower and the organisms were pleomorphic but large amounts of toxin were produced. Therefore, we assume that sub-optimal growth conditions result in pleomorphic cells and a large toxin yield.

Sodium deoxycholate, a surfactant, was used to determine the effect of bile salts on *V. cholerae*. Its ability to inhibit the aerobic growth of *V. cholerae* is interesting since aerobic Gram-negative bacteria are resistant to the inhibitory action of bile salts (Binder, Filburn & Floch, 1975). Sodium deoxycholate did not affect the toxin yield per mg dry wt of culture under sub-optimal growth conditions, i.e. aerobically at 30°C or anaerobically at 37°C, whereas the toxin yield was increased in cultures incubated aerobically at 37°C from 0.1 to 5.0 µg ml⁻¹, which was equal to or greater than the toxin yield under sub-optimal conditions. Other surfactants such as sodium glycocholate, the most common conjugated bile salt of man, and the non-ionic detergent Brij-38 also increased toxin yield in aerobic cultures at 37°C. Sodium glycocholate, unlike sodium deoxycholate, did not inhibit the growth of *V. cholerae*. Floch *et al.* (1972) obtained similar results using other intestinal organisms.

The ability of sodium deoxycholate to increase toxin yield in cultures grown aerobically at 37°C was not due to activation of 'free' toxin molecules since it did not increase the activity of crude cholera toxin. Nor was it due to release of intracellular toxin since mechanical lysis of organisms grown aerobically at 37°C yielded little toxin. However, treatment of the mechanically lysed cells or of whole cells grown aerobically at 37°C with 0.1 % sodium deoxycholate released large amounts of toxin, indicating that the toxin was bound to some component of the cell. Tris/EDTA, which releases periplasmic enzymes from

Gram-negative bacteria (Leive & Shovlin, 1968), did not release active toxin but did release biologically inactive antigen which cross-reacted with the toxin in the PHI test. This may be of some practical interest since the non-toxic antigen is likely to be choleraegenoid, a naturally occurring non-toxic antigen (Finkelstein, Peterson & LoSpalluto, 1971).

Our results can account for the physiology of enterotoxin secretion. The organism produces toxin under a variety of cultural conditions, and this toxin is bound, probably to a lipid-containing component of the cell. If the environmental conditions result in abnormal morphological forms – an outward manifestation of altered structure and function of the cell envelope – the toxin escapes to the environment. When the morphology is 'normal' the toxin is bound and can only be released when the cell envelope is ruptured and the toxin is released from the lipid (by using detergents such as bile salts). This may be similar to the results reported by Evans, Evans & Gorbach (1974) on the release of enterotoxin from *E. coli* by treatment with polymyxin B.

Our experiments were directed to learning more about cholera enterotoxin production under conditions that resembled those found in the human intestine rather than under the highly aerobic conditions at 30°C used in the conventional *in vitro* methods. It has been shown that cholera vibrios can produce extracellular toxin when grown at 37°C under anaerobic conditions or aerobically at 37°C in the presence of bile salts, especially sodium deoxycholate. The experiments indicate that one does not need the large cell yield obtained from aerobic cultures to get an equivalent quantity of toxin from anaerobic cultures. To explain how sodium deoxycholate becomes involved in cholera pathogenesis, one needs only to recall that this is formed by the action of intestinal bacteria on primary bile acids, and the upper small bowels of cholera patients have been found to be colonized by a number of bacteria capable of effecting this chemical change (Gorbach *et al.*, 1970).

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REFERENCES

- ARANKI, A. & FRETER, R. (1972). Use of anaerobic glove boxes for the cultivation of strictly anaerobic bacteria. *American Journal of Clinical Nutrition* **25**, 1329–1334.
- BANERJEE, D. N. (1939). Culture du *Vibron cholérique* anaérobie, les variations de son pH et son pouvoir toxique. *Comptes rendus des séances de la Société de biologie* **130**, 32–34.
- BINDER, H. J., FILBURN, B. & FLOCH, M. (1975). Bile and inhibition of intestinal anaerobic organisms. *American Journal of Clinical Nutrition* **58**, 119–125.
- BURROWS, W. & KAUR, J. (1974). Cholera toxins. In *Cholera*, pp. 143–167. Edited by D. Barua and W. Burrows. Philadelphia: W. B. Saunders.
- CALLAHAN, L. T., RYDER, R. C. & RICHARDSON, S. H. (1971). Biochemistry of *Vibrio cholerae* virulence. II. Skin permeability factor/cholera enterotoxin production in a chemically defined medium. *Infection and Immunity* **4**, 611–618.
- CRAIG, J. P. (1966). Preparation of the vascular permeability factor from *Vibrio cholerae*. *Journal of Bacteriology* **95**, 793–795.
- DE, S. N., GHOSH, M. L. & CHANDRA, J. (1962). Further observations on cholera enterotoxin. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **56**, 241–245.
- EVANS, D. J., EVANS, D. G. & GORBACH, S. L. (1974). Polymyxin B induced release of low molecular weight heat-labile enterotoxin from *Escherichia coli*. *Infection and Immunity* **10**, 1010–1017.
- FINKELSTEIN, R. A. (1970). Monospecific equine antiserum against cholera enterotoxin. *Infection and Immunity* **2**, 691–697.
- FINKELSTEIN, R. A., ATTHASAMPUNNA, P., CHULASAMAYA, P. & CHARUNMETHEE, P. (1966). Pathogenesis of experimental cholera: biological activities of purified procholeraegen A. *Journal of Immunology* **96**, 440–449.

- FINKELSTEIN, R. A., PETERSON J. W. & LoSPALLUTO, J. J. (1971). Conversion of cholera exo-enterotoxin (choleragen) to natural toxoid (choleragenoid). *Journal of Immunology* **106**, 868.
- FINKELSTEIN, R. A. & PETERSON, J. W. (1970). *In vitro* detection of antibody to cholera enterotoxin in cholera patients and laboratory animals. *Infection and Immunity* **1**, 21-29.
- FLOCH, M. H., BINDER, H. J., FILBURN, B. & GERSHENGORAN, W. (1972). The effect of bile acids on intestinal microflora. *American Journal of Clinical Nutrition* **55**, 1418-1426.
- FRETER, R. (1969). Studies of the mechanism of action of intestinal antibody in experimental cholera. *Texas Reports on Biology and Medicine* **27**, Suppl. 1, 299-316.
- FRETER, R. (1972). Parameters affecting the association of vibrios with the intestinal surface - experimental cholera. *Infection and Immunity* **6**, 134-141.
- GALLUT, J. & JUDE, A. (1955). Contributions a l'étude de la virulence et du pouvoir toxigène du *Vibrio cholérique*. II. Influence de la température d'incubation sur le pouvoir toxigène *in vitro* de *Vibrio cholerae* (Ogawa). *Annales de l'Institut Pasteur* **88**, 282-288.
- GORBACH, S. L., BANWELL, J. G., JACOBS, B., CHATTERJEE, B. D., MITRA, R., BRIGHAM, K. L. & NEOGY, K. N. (1970). Intestinal microflora in Asiatic cholera. II. The small bowel. *Journal of Infectious Diseases* **121**, 38-45.
- HIRSCH, J. VON (1926). Die anaerobe Züchtung des *Vibrio cholerae*. *Klinische Wochenschrift* **5**, 1089.
- HOFMANN, A. F. (1962). Thin-layer adsorption chromatography of free and conjugated bile acids on silicic acid. *Journal of Lipid Research* **3**, 127-128.
- KASAI, G. J. & BURROWS, W. (1966). The titration of cholera toxin and antitoxin in the rabbit ileal loop. *Journal of Infectious Diseases* **116**, 606-614.
- KENNEDY, J. R. & RICHARDSON, S. H. (1969). Fine structure of *Vibrio cholerae* during toxin production. *Journal of Bacteriology* **100**, 1393-1401.
- KUSAMA, H. & CRAIG, J. P. (1970). Production of biologically active substances by two strains of *Vibrio cholerae*. *Infection and Immunity* **1**, 80-87.
- LEIVE, L. & SHOVLIN, V. K. (1968). Physical, chemical and immunological properties of lipopolysaccharide released from *Escherichia coli* by ethylenediaminetetraacetate. *Journal of Biological Chemistry* **543**, 6384-6391.
- LINTON, R. W., MITRA, B. N. & SEAL, S. C. (1936). Further notes on the cholera and cholera-like vibrios. *Indian Journal of Medical Research* **23**, 601-607.
- MILES, A. A., & MISRA, S. S. (1938). The estimation of the bactericidal properties of blood. *Journal of Hygiene (Cambridge)* **38**, 732-749.
- NORMAN, A. & GRUBB, R. (1955). Hydrolysis of conjugated bile acids by clostridia and enterococci. *Acta pathologica et microbiologica scandinavica* **36**, 537-547.
- PIERCE, N. F. & WALLACE, C. K. (1972). Stimulation of jejunal secretion by a crude *Escherichia coli* enterotoxin. *Gastroenterology* **63**, 439-448.
- POLLITZER, R. (1959). *Cholera*. World Health Organization monograph series no. 43. Geneva, Switzerland: WHO.
- RICHARDSON, S. H. (1969). Factors influencing *in vitro* skin permeability factor production by *Vibrio cholerae*. *Journal of Bacteriology* **100**, 27-34.
- RICHARDSON, S. H., EVANS, D. G. & FEELEY, J. C. (1970). Biochemistry of *Vibrio cholerae* virulence. I. Purification and biochemical properties of PF/cholera enterotoxin. *Infection and Immunity* **1**, 546-554.