Special Issue on S. N. De and
Cholera Enterotoxin

Some Aspects of Cholera Research

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A number of Indian scientists have contributed significantly in different areas of research on cholera. S. N. De's contribution is unique in the sense that it resolved the age old controversy between the endotoxin and exotoxin as the causative factor of the disease. Since De's publication[1,2], it has been resolved that cholera toxin is an exotoxin and all subsequent investigations are based on this concept. Another significant contribution of De is the development of a model for assay of cholera toxin, i.e., the rabbit ileal loop model[3]. I had the experience of working almost next door (Calcutta School of Tropical Medicine) to De's laboratory at the Calcutta Medical College and interacting with him on areas of mutual interest. In this context, I feel it is a unique privilege for me to be able to write on cholera as a tribute to S. N. De.

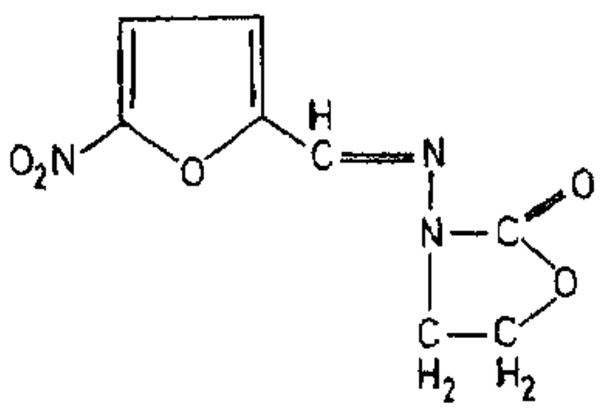
In this article I present a brief sketch of the problems and prospects of the disease cholera as overviewed by a basic scientist[4]. I do not pretend to present here an elaborate treatment of all the different facets of research on cholera but shall try to present an overview on some specific problems. I shall at the same time elaborate on those areas where I have had a personal interest.

The cholera victim ingests viable *Vibrio cholerae* through contaminated drinks or food. The bacteria multiply in the small bowel and secrete the enterotoxin. The choleragenic toxin then interacts with the membranes bounding the intestinal mucosal cell layers and activates the membrane bound enzyme, adenylate cyclase. The resulting accumulation of cyclic AMP in the intestinal mucosa leads to the severe fluid loss characteristic of cholera. As a direct result of this fluid loss, the severely ill cholera patient rapidly develops hypovolemic shock. Also as a result of this intestinal fluid loss, the patient rapidly develops an increased concentration of plasma protein and formed elements of the blood. Further, because of extensive loss of potassium and bicarbonate along with the cholera stool, the typical rice-water stool, the total body potassium depletion and severe metabolic acidosis result. Cholera is often fatal and the major causes of death are hypovolemic shock, renal failure and uncompensated metabolic acidosis.

The causative agent of the disease cholera is generally the bacterium, *Vibrio cholerae*. Unlike the bacterium, *Escherichia coli*, *V. cholerae* organisms had been much less studied and accordingly extensive characterization of *V. cholerae* has been an important and major area of research. This includes investigation of the cholera bacteriophages, since bacteriophages are often used as tools for gaining information on bacteria. I shall deal with the characterization of vibrios, particularly those areas wherein we involved ourselves, in greater detail. I shall not deal with the vibriophages here since a number of review articles have adequately dealt with the subject[5–7]. It appears that a reliable phage typing for *V. cholerae* Ol is yet to be achieved[8]. In this respect electron microscopic monitoring of the typing phages should be considered as an important proposition. Application of classical genetics, as initiated by Bhaskaran[9,10], and also recombinant DNA technology for a thorough characterization of the genetic resources of the organism and also for deriving a non-toxinogenic strain of *V. cholerae* which may be used as a live oral vaccine, should provide enough challenge to future researchers. The genetically engineered non-toxinogenic strain of *V. cholerae* is expected to provide local immunity at the small intestine against cholera infection. This area is being actively pursued at this moment both from the fundamental as well as applied aspects of the problem[7]. Other modes of immunization against cholera are still open to further research. Another area of practical importance is the development of the oral rehydration fluid, a sterile, pyrogen-free fluid of appropriate sodium, potassium, chloride, bicarbonate and glucose content to be administered orally to a

cholera patient. The oral therapy has proved to be effective and practical in hospitals and in the field in endemic areas. However further research is needed for elucidating the actual mechanism of rehydration by oral administration of such a fluid.

In the area of cholera therapy, the best compromise now appears to be to provide speedy replacement of the salts and water lost in the diarrhoeal stool and at the same time to take recourse to antibiotic treatment for prompt killing of the vibrios and hence prevention of further toxin production. Tetracycline has been mostly the antibiotic of use in cholera therapy. Furazolidone, a synthetic nitrofuran



FURAZOLIDONE

[N-(5-nitro-2-furfurylidene)-3-amino-2-oxazolidone]

drug was found by several investigators[11-13] to be effective in cholera cases as an alternative to tetracycline. Furazolidone, from points of view of classical pharmacology, was till then known to be much less toxic than tetracycline, although the mechanism of action of furazolidone at the molecular level, unlike tetracycline, was practically unknown. We have investigated this aspect and I shall deal with the same subsequently with greater detail. The cholera toxin has now been fairly well characterized. But another area providing scope for further investigation appears to be the interaction between cholera enterotoxin and adsorbants, ligands etc. with the practical objective to spare the cell membrane of the intestinal mucosal layer and the subsequent fluid loss. Although this field has not been extensively investigated, recent studies have revealed that the cholera enterotoxin binds to the G_{M1} molecules[14] present on the outer surface of the plasma membrane of cells lining the intestinal lumen. It has also been documented that sialic acid[15] is required for cholera toxin to bind to G_{M1} and that the B sub-units of cholera toxin are responsible for the binding to cell membrane. In an elegant study Schengrund and Ringler[16] showed that a non-lipid compound containing polyvalent clusters of G_{M1}, i.e., oligo-G_{M1}-poly-L-lysine, is likely to compete effectively with the cell membrane G_{M1} sites and block the binding of toxin to membrane G_{M1}.

Characterization of the Vibrios

Fimbriae. Fimbriae or pilli projecting from the surface of *V. cholerae* (*eltor*, and classical) strains as long thin filaments of axial diameter ranging between 60 and 100 Å were detected by electron microscopy[17,18] in all the haemagglutinating strains examined. The general features of these fimbriae are that their number per bacterium is very small, mostly less than ten, and that very often they are not straight. One *V. cholerae eltor* strain was, however, found to possess a larger number of more or less straight fimbriae on one side of its lateral surface[19]. Fimbriated *eltor* vibrios were found to promote pellicle formation on the surface of the static liquid cultures and some sort of correlation between the degree of fimbriation and the minimum time needed to form the pellicle was indicated.

Fimbriated *eltor* vibrios were found to agglutinate red blood cells of man and different animals. The haemagglutination reaction was however much weaker for *eltor* vibrios than for *E. coli* in the fimbriate phase[17]. If the fimbriae are assumed to be the haemagglutinating agents of *eltor* vibrios, the weaker reaction can be explained as due to the presence of smaller number of fimbriae per organism present in the culture. The observations which support the assumption that fimbriae on *eltor* vibrios are the haemagglutinating agents are: (i) bacteria-free culture supernatant do not agglutinate red blood cells[18,20], (ii) D-mannose partially inhibits haemagglutination[17,20], (iii) haemagglutination reaction is strongest when the organisms are most fimbriated[18], (iv) the organisms are not eluted from red cells with the rise of temperature[19], (v) the organisms do not haemagglutinate at 4°C[20] and (vi) the fimbriated strains form the pellicle, i.e., the organisms adhere to each other when they possess fimbriae[19]. As against the above evidences, the observations which may suggest that the *eltor* haemagglutination is nonfimbrial are: (i) there is no significant change in haemagglutination reaction after serial passages in nutrient agar[21] and (ii) the reaction is inhibited by formalin, phenol and ethanol[22]. Electron microscopic evidences have consistently shown that fimbriae on *eltor* vibrios are atypical and apparently do not belong to any one of the types described by Brinton[23] or by Duguid *et al*[24]. Also it is not known whether all the different types of fimbriated bacteria are equally affected by formalin, phenol and ethanol. The relation between fimbriae and haemagglutinating activity of vibrios remains undecided. Sex fimbriae or pilli were detected by Bhaskaran *et al*[25] on some conjugating male strains of *V. cholerae*.

V. cholerae is now known to produce at least four

distinct haemagglutinins[8]. Cell associated and soluble haemagglutinins are present in strains of both biotypes. The cell bound haemagglutinins of *V. cholerae* Ol may be responsible for mucosal attachment and act as vibrio adhesins[8]. Other factors related to the virulence of vibrios are chemotaxis and motility. Intestinal epithelium is known to contain strong chemotactic factors (taxins) to which *V. cholerae* Ol can respond. Also it has been shown that mutants lacking taxin receptors have reduced virulence[8]. There is scope for further research in these areas.

Flagellum. Electron microscopy revealed the presence of a single polar flagellum in *V. cholerae* cells[26,27]. The flagella in vibrios are usually sheathed and of overall thickness 300 Å. The flagellar core, exposed after autolysis of the cells, measures 150–170Å in width. Negative staining revealed the presence of a narrow and hollow axial region of width about 30Å. Electron microscopy suggested a continuity between the flagellar sheath and the cell wall of vibrios. Subsequent study by immunoelectron microscopy demonstrated the presence of lipopolysaccharide on sheathed flagella of *V. cholerae* Ol[28]. The flagellar core was however found to pass through the plasma membrane and get attached or anchored to a structure, usually called basal granule, at the polar region of the bacterial cytoplasm. The dimension of the basal granule, which looked like a cup-shaped structure in negatively stained preparations, was about 640 Å. The flagellum provides motility to the vibrios.

Ultrastructure of cell wall and plasma membrane. Electron microscopy of the ultra thin section of *V. cholerae* classical and *V. cholerae eltor* in their active phase of growth consistently revealed a triple layered structure[29–31] (dark-light-dark) of the cell wall of thickness about 100 Å. The thickness of individual layers ranged between 30 and 33 Å. This observation was in contradiction with findings of the five layered structure of the cell wall in *E. coli*[32], *Thiobacillus thioxidans*[33] and some strains of unusually stalked bacteria[34]. However, the cell walls of all gram-negative bacteria do not reveal the presence of five layers by electron microscopy and cell walls of *Cytophaga marinoflava*[35], *Spirillum serpens*[32] and *Vibrio fetus*[36] showed the presence of three layers only. It may be that the mucopeptide layer in these organisms including *V. cholerae* has very weak or no affinity to the electron stains in use. In fact the isolated mucopeptide layer of *V. cholerae* cell wall could be detected by metal shadowing and electron microscopy[37]. Also isolated cell walls of *V. cholerae* did not reveal the presence of any fine structure, e.g., regular array of globular units etc. Immediately beneath the triple layered cell wall structure, a much more electron transparent layer of thickness varying between 50 and 100 Å could be detected. This layer separated the outer triple layered cell wall from the plasma membrane, which also exhibited three layers of overall thickness between 70 and 90 Å.

Ultrastructure of the *V. cholerae* cell during autolysis was also investigated. During autolysis of the vibrios, the plasma membrane often retracts unevenly from the cell wall and their normal relative disposition is destroyed[26]. Gallut and Giuntini[38] studied the *V. cholerae* lysis by different methods and concluded that the cell wall dissolved during autolysis. Autolysed cells of *V. cholerae* examined by electron microscopy[30] resolved, on the other hand, the presence of both plasma membrane (considerably retracted) and cell wall, in agreement with the observations of many authors on other gram-negative bacteria. However, leeching out of the chemical components of the wall could not be ruled out.

Cytoplasm and nucleus. Electron microscopic cytology of *V. cholerae* was typical of any gram-negative bacterium[39]. The cytoplasm is studded with electron dense materials, mostly ribosomal particles. The nuclear zone is considerably electron transparent, distributed and often crossed by cytoplasmic bridges. The nucleoplasm is not bounded by any membrane. Under high resolution electron microscopy, the nucleoplasm revealed the presence of DNA filaments of about 20 Å in diameter[31]. The ultrastructure of cell division was found typical of a gram-negative bacterium.

***V. cholerae* DNA.** Physico-chemical studies[40–41] of isolated chromosomal DNA of *V. cholerae* revealed a total phosphorus content of 9.23% (w/w) and extinction coefficient with respect to phosphorus, ε_p as 6405 at 260 nm. The G-C content as estimated from melting temperature and by other methods is 48 mole%, the corresponding melting temperature in 1× SSC being 89°C by d-assay and 90.5°C by i-assay methods. During thermal transition in 1 × SSC (pH 7.1), *V. cholerae* DNA exhibited an hyperchromicity of 39% and the degree of co-operativeness, $\Delta T_{2/3}$ as 3.9. Study of differential melting curves of *V. cholerae* chromosomal DNA revealed a unimodal distribution of G-C base pairs. The molecular weight of the chromosomal DNA measured $3.29 \pm 0.38 \times 10^9$ daltons[42]. 51% (v/v) methanol in 5 mM tris-HCl buffer (pH 7.4) lowered the melting temperature to 48.5°C and 1% (v/v) formaldehyde in 5mM NaCl (pH 6.8) to 56.5°C. Copper ions have a drastic effect on stability of *V. cholerae* genome DNA[43]. 100 μM Cu (II) ions in 5 mM NaCl (pH 6.8) lowered the melting temperature from 72°C (control) to 33°C. The effect of copper ions on the viability of *V. cholerae* cells may provide scope for further study.

Few plasmids have so far been detected in *V. cholerae* cells[7]. Male strains of *V. cholerae* investigated so far contain a plasmid sex factor, P[9]. This sex plasmid cannot integrate stably into the host chromosome to form high frequency recombinant strains presumably because of a significant difference of G-C content in the chromosomal and plasmid DNA[7,44] (48 and 42% respectively). The size of the sex plasmid was found to vary between 47×10^6 and 80×10^6 daltons. Restriction analysis as well as electron microscopic measurements demonstrated a length of 68 kilobases or 45×10^6 daltons. Two additional plasmids, 4.7 and 34 kb in size, were detected in the Bhaskaran strain 162[44,45]. Their functions are not known. Drug resistance in *V. cholerae* is supposed to be mediated by the so-called R-factor plasmids. Genetics of *V. cholerae* and cholera bacteriophages have recently been reviewed by Guidolin and Manning[7] and hence this aspect will not be elaborated here any further.

Action of surface active agents. Studies on the sensitivity of different bacteria to surface active agents are likely to throw light on the physico-chemical structure of their surface layers and particularly the cell wall[46]. EDTA in presence of tris buffer caused marked lysis of the vibrios[47], which could be inhibited by a number of divalent and monovalent cations and also by sucrose. On addition of lysozyme to the tris + EDTA system, a greater degree of lysis was obtained. Also sodium lauryl sulphate caused a greater degree of lysis. A considerable amount of protein, lipid, nucleic acid and carbohydrate materials were found to be released after EDTA treatment. EDTA-induced lysis was dependent on its concentration and maximum lysis was attained at EDTA concentration of 50 μg/ml. Similar observations on the EDTA-induced lysis of *V. succinogenes*[48] and *Pseudomonas aeruginosa*[49] were reported by several investigators.

EDTA also caused a marked loss of viability of vibrios, the extent of loss being dependent on EDTA concentration. On the basis of loss of viability, EDTA action could differentiate the *V. cholerae* classical and *V. cholerae eltor* biotypes. Ten minutes treatment with 10 μg EDTA/ml left 43–50% of *eltor* vibrios and 71–75% of classical strains viable[47]. EDTA toxicity was also reported to be sex differentiating in *E. coli*[50], male strains possessing sex fimbriae being less viable than the female ones. EDTA sensitive bacteria are supposed to possess surface layers which are easily altered by EDTA treatment, allow the chelating agent to enter the cell readily and damage sensitive intracellular targets. In fact there are reports indicating the toxic effect of EDTA in the functioning of chromosomes and RNA[51], on the inactivation of metalloenzymes and on causing death by metal starvation[52]. Also the presence of fimbriae on *eltor* strain might contribute to their greater sensitivity to EDTA. Wilkinson[53] also reported that the loss of viability is a sensitive index for differentiation of EDTA sensitive and resistant organisms.

When the actively growing vibrios are exposed to ethanol, pool materials or metabolic intermediates are released very quickly. Labelled organisms exhibited maximum leakage of ^{32}P-compounds when exposed to 71% (v/v) ethanol for *V. cholerae* classical and 50% (v/v) ethanol for *V. cholerae eltor*[37,47], leakage in 100% ethanol was about 77% of maximum for *V. cholerae* classical and 66% for *V. cholerae eltor*. The nature of release of ^{32}P-compounds by bacteria suspended in ethanol reveals the physico-chemical state of their cell wall, the passage of small molecules being impeded differently in different bacteria[54,55]. Salton[54,55] also suggested a general correlation between the degree of release of ^{32}P-compounds and the amount of mucopeptide component in the respective cell wall.

Isolated cell wall: Physico-chemical study. Purified cell wall fractions of *V. cholerae* grown in peptone-water medium were subjected to various physical and chemical investigations[30]. The isolated cell wall fraction contained protein 42.3%, total carbohydrate 9.2%, hexose 5.2%, hexosamine 3.5%, lipid 18%, phosphorus 1.42%, calcium 0.18% and magnesium 0.12% by weight. Isolated cell wall fraction presented infra red absorption maxima at 1120, 1160, 1240, 1300, 1380, 1450, 1540, 1655, 2850 and 3250 cm^{-1}. Molecular weights of the protein units in the isolated cell wall, as obtained by the SDS-PAGE technique, were (i) 167000, (ii) 155000, (iii) 128000, (iv) 108000, (v) 83000, (vi) 70000, (vii) 57000, (viii) 53000, (ix) 45000, (x) 35000, (xi) 25000, (xii) 20000 and (xiii) 13000 daltons. The 13000 protein unit constituted the major fraction among the cell wall proteins. These observations substantiated the views of Schniatman[56] that the presence of one or few major protein species could be a common feature of all gram-negative bacterial cell walls. Isolated cell walls of *V. cholerae* contained receptors of the cholera phage ϕ 149 (Mukherjee's Gr. IV). Treatment with sodium deoxycholate inactivated the phage receptors in the cell wall[60]. Different investigators[61,62] also reported the presence of ten or more proteins in the *V. cholerae* outer membrane fraction, of which the 45000 dalton proteins were most probably porins, the 35000 dalton protein was shown to behave like the *Omp A* protein of members of the family *Enterobacteriaceae*[63]. Kelley and Parker[61] observed that the 21000 dalton protein in the *V. cholerae* outer membrane could represent the cholera toxin subunit

Al in conformity with the idea of Fernandes *et al*[64] that cholera toxin is possibly membrane bound or assembled at the membrane level before secretion. The profile of membrane proteins was found to vary with media and culture conditions[62]. The osmolarity of the growth medium was also reported to modulate the expression of outer membrane proteins in *V. cholerae*[65]. Variations in protein composition among different vibrio species were also demonstrated[66]. The gene for a very immunogenic outer membrane protein has been cloned and termed *omp V*[7]. The *omp V* protein was identified as the one having molecular weight around 26000 daltons. The *omp V* protein was found in all *V. cholerae* strains investigated irrespective of their biotype or serotype, but was not found in other vibrios such as *V. mimicus* and *V. fluvialis*[7]. Further work is needed to decide whether *omp V* protein is a protective antigen. The 13000 dalton protein was greatly enhanced under anaerobic growth condition[62]. The presence of a common (to biotypes and serotypes) protein antigen in the outer membrane of *V. cholerae* was suggested.

Chemical and immunological aspects of *V. cholerae* lipopolysaccharide (LPS) have been investigated extensively[7]. It may however be noted that cholera LPS was found to contain several unusual sugars, e.g., 2-amino-2, 6-dideoxy-D-glucose (quinovosamine), 4-amino-4-deoxy-L-arabinose etc. Cholera LPS, like LPS of other gram-negative bacteria, contains lipid-A, a core region and O-antigenic side chains. Unlike *E. coli* and *Salmonella* species, *V. cholerae* possesses only a single 2-keto-3-deoxyoctulusonic acid in its core. Phage adsorption studies revealed the presence of receptors of group IV cholera phage ϕ 149 in LPS of the classical biotype but not in LPS of *eltor* biotype[60]. Treatment with sodium deoxycholate dissociated the LPS and inactivated the phage receptors in conformity with the observations on the LPS in *Salmonella minnesota*[67].

Microanatomy of a novel secretory activity of *V. cholerae*. Thin sections of *V. cholerae* harvested during the logarithmic phase of growth in alkaline peptone water or in syncase medium revealed an excretion process of the cell wall in the form of bulging out and pinching-off of portions[39]. Particles closely resembling the pinched off cell wall structures were detected by electron microscopy of the bacteria free filtrate of the log phase cultures. The sac like structures measured 400–1100 Å in diameter. An interesting feature of the actively growing cells was the presence of finer particles of size between 40 and 100 Å, lying in association with the cell wall or surface blebs or lying immediately outside the cell surface where apparently no bleb like structures could be seen. Surface blebs could also be detected by the negative staining and electron microscopy of the bacterial culture[31]. Also the finer 40 – 100 Å particles and the bigger sac like structures could be detected in the extensively dialysed log phase culture filtrate of *V. cholerae*[31,68]. It was interpreted that bleb formation represented an excretory mechanism of *V. cholerae*, a mechanism not reported beforehand for any bacterial cell. It was imagined that the finer 40 – 100 Å particles could represent the cholera enterotoxin, although their functional identity was not established[31,68]. Kennedy and Richardson[69] recorded observations which were not in agreement with (i) the reports of Chatterjee and Das[39] on the formation of surface blebs of *V. cholerae* cells and (ii) the results of Kusama and Craig[70] in respect of the kinetics of bacterial growth and toxin production. Many subsequent investigators[71–73], however, confirmed the formation of surface blebs on *V. cholerae* as an active secretory mechanism in vibrios, simulating reverse pinocytosis, whereby non diffusible substances could be removed from the cell. Pike and Chandler[74,75] in fact presented evidence that heat stable somatic antigens are present in the culture fluid of *V. cholerae* primarily as a result of spontaneous release rather than as a consequence of bacterial lysis. The formation of surface blebs described by Chatterjee and Das[39] could easily account for the spontaneous release of somatic antigens. A correlated biochemical and electron microscopic study of the growth of *V. cholerae* cells was reported by Chatterjee *et al*[31]. Actively growing cells were found to excrete non dialysable materials containing protein, carbohydrate and fatty acids. At least six different protein bands could be detected by polyacrylamide gel electrophoresis. RNase, DNase, lipopolysaccharide and somatic antigens were detected in log phase culture filtrate[31]. Blebs and finger like protrusions were found on the surface of different strains of *V. cholerae* irrespective of the method of fixation used for electron microscopy[31].

Another observation of interest was that extra amounts of carbohydrate and protein were released when the *V. cholerae* cells were subjected to cold shock[31]. Similar effects of cold shock on *E. coli*[76], *P. aeruginosa*[77], *S. typhimurium*[77] etc. occurring presumably due to an initial damage in plasma membrane were reported by different investigators. For study of the excretion process of *V. cholerae*, chilling of the culture, immediately after growth attains a desired stage, should thus be avoided. The rate and extent of lysis of *V. cholerae* cells under non growing conditions were also reported to depend on osmolarity of the growth medium[65]. It was observed that the behaviour of *V. cholerae* is atypical as far as its response to osmotic stress is concerned and re-

MICROANATOMY OF *VIBRIO CHOLERAE* REVISITED

The electron micrographs presented here were recorded in the author's then laboratory at the Calcutta School of Tropical Medicine during the period between 1961 and 1970 and in course of investigations on cholera on a research project financed by the Indian Council of Medical Research. All the micrographs were obtained on a HITACHI HS-6 electron microscope providing maximum instrumental magnification of 20,000x and resolution 20 Å.

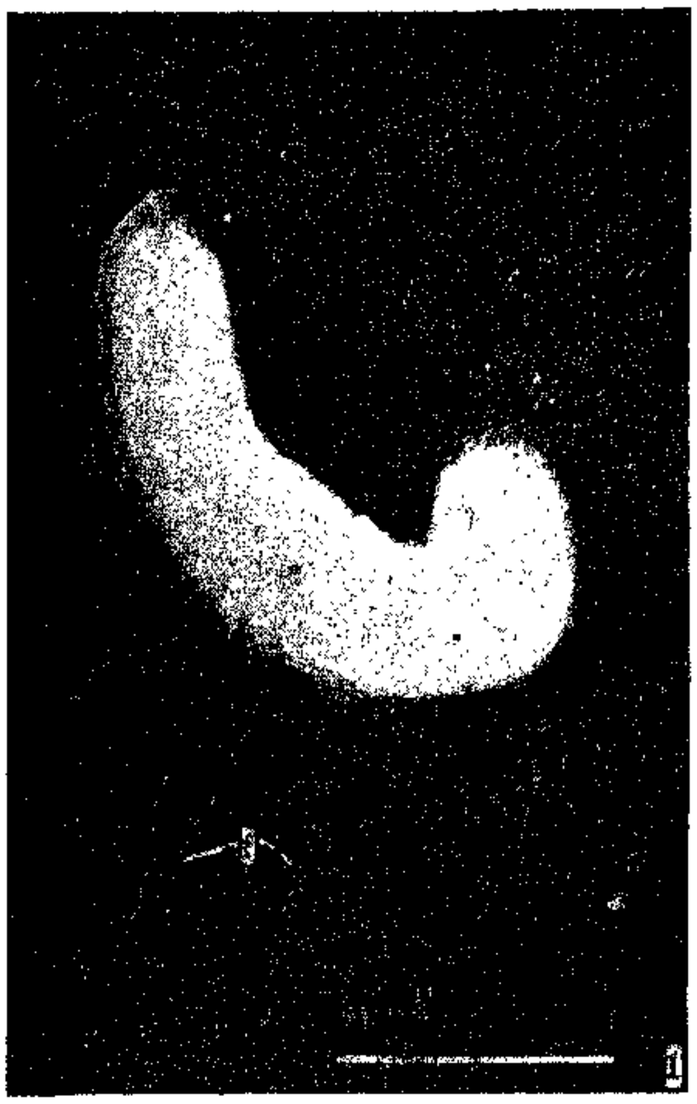


Figure 1. A typical comma shaped *V. cholerae ehor* strain OE/27 shadowed with chromium. The presence of long thin fimbriae (f) on one side of the lateral surface of the bacterium can be seen. This type of fimbrial disposition is very atypical and not found in other bacteria. White bar represents 1.0μ.

Figure 2. **(a)** Ultra thin section of part of one *V. cholerae* cell demonstrating the presence of very thin DNA filaments in the nuclear zones (n). Bar represents 0.1μ.
(b) Ultra thin section of part of a V. cholerae cell and portions of flagellum (F). The flagellar sheath and core regions can be seen clearly. Bar represents 0.1μ.
(c) Ultra thin section of part of a *V. cholerae* cell at higher magnification demonstrating the trilamellar structures of the cell wall (CW) and plasma membrane (PM). Bar represents 0.1μ.
(d) Negatively stained (by phosphotungstic acid) preparation of an autolysed flagellum showing the separated sheath (sh) and the core filament (f). Broken fragments (bf) of the core can also be seen at different places. Bar represents 0.1μ.

sembles that of halobacteria or other gram-negative bacteria whose peptidoglycan constituent has been damaged by lysozyme or antibiotics. *V. cholerae* cells were also shown to undergo interesting morphological changes, conversion from rod shaped structure to spherical protoplast like structures, when incubated in a glucose-saline medium[78].

Recent studies have elucidated that the *V. cholerae* enterotoxin is an oligomeric protein comprising of six sub units, an A sub-unit (28 kilodalton) that activates adenylate cyclase and five B sub-units (12 kilodaltons each) which bind to G_{M1} ganglioside receptors[16,79,80]. If the holotoxin of 88 kilodalton size is synthesized within the bacterial cell, it is difficult to imagine how such a molecule can pass through the plasma membrane and the cell wall in order to be excreted into the extracellular medium. Electron microscopic studies[31,39] suggested an active process for secretion of such protein molecules. Although any alternative excretion or secretion process is not yet adequately known, recent evidences have agreed with earlier observations that there must exist an active process of the *V. cholerae* cell wall membrane for effecting toxin secretion by the actively growing vibrios[80]. It now appears that the toxin subunits enter the bacterial periplasm prior to their secretion into the medium. Also available evidence indicates that the enterotoxin subunits indeed assemble within the periplasm before they are secreted[80]. Recent studies have further suggested that the outer membranes of vibrio species possess a property or properties which permit toxin to be translocated through them[80]. Genetic analysis[7] has however suggested that *V. cholerae* has more than one excretion system and that those systems have a high degree of selectivity in the proteins which they release.

Antimicrobials in the Treatment of Cholera. Molecular Mechanism of Action and Genotoxicity of Furazolidone

As stated earlier the broad spectrum antimicrobial synthetic nitrofuran drug, furazolidone was found to work almost as effectively as tetracycline and, in accordance with classical pharmacology, was much less toxic[11-13,81]. Unfortunately the mode of action of furazolidone, particularly at the molecular level, *vis-a-vis* tetracycline, was practically unexplored. This prompted us to undertake investigations on the molecular mechanism of action and genotoxicity, if any, of furazolidone. Since bacteriological tests showed that *V. cholerae* is very sensitive to the action of furazolidone[11,12] and since the use of furazolidone in the treatment of cholera cases was the factor which initiated our investigation, we used the *V. cholerae* cell as the model for the study of drug action. However, furazolidone or N-(5-nitro-2-furfurylidene)-3-amino-2-oxazolidone was already known to exhibit a wide spectrum of antimicrobial activity (besides *V. cholerae*) and had found useful application in human therapy, e.g., treatment of gastrointestinal and urino-genital infections[82-84].

Inhibition of DNA biosynthesis in *V. cholerae* by furazolidone. Furazolidone at the concentration level of 0.5μg/ml inhibited DNA synthesis very significantly, but increased, as compared with the control, the RNA and protein synthesis per equal growth[85,86]. At this level of drug concentration, the colony forming capacity of the vibrios gradually decreased to 25% of the initial value and thereafter remained steady. With the increase of furazolidone concentration beyond 0.5μg/ml, there was a progressive decrease in the synthesis of RNA, DNA and protein. At the concentration level of 5.0μg/ml, furazolidone caused a very rapid loss of viability of the *V. cholerae* cells and the protein, RNA and DNA contents in the cell became considerably less than the respective initial values, indicating degradation and release of these components in the extracellular medium.

Filamentation of *V. cholerae* cells. In conformity with the biochemical findings on macromolecular synthesis, electron microscopic as well as light microscopic studies revealed that the *V. cholerae* cells underwent filamentation following furazolidone (0.5μg/ml) treatment[85,86]. The lengths of the filamentous cells varied between 6 and 11 times that of the untreated or native ones. In some modes of drug treatment, a significant number of cells grew into extremely long filamentous forms[87] of lengths even greater than 40μm (20 times the length of a native cell). Treatment with antiserum prepared against heat killed vibrios (non-treated with drug) resulted in a significantly faster agglutination of the filamentous cells as compared with the untreated ones. After treatment with 5.0μg/ml of furazolidone, the cells were approximately of normal shape and size but became considerably electron transparent due to leakage of materials.

Furazolidone on phage infected vibrios. Furazolidone in concentrations which had little effect on growth of host organism greatly reduced the yield of phage ϕ 149 (cholera phage belonging to Mukherjee's group IV) from the host *V. cholerae* strain OGAWA 154[88,89]. The phage was resistant to the *in vitro* action of the drug in dark. The average burst size of the drug treated and infected bacteria decreased exponentially with increase in drug concentration. Furazolidone at the concentration level 0.05μg/ml inhibited DNA synthesis by about 50% in phage infected cells and only by about 18% in non-infected ones, relative to the respective controls. RNA and protein synthesis were affected by a much smaller degree both in infected and non infected cells. Phase receptors on the surface of the host cells were practically unaffected after furazolidone treatment.

Molecular basis of the inhibition of DNA biosynthesis by furazolidone: With a view to explaining the molecular basis of the inhibition of DNA biosynthesis, the *in vitro* interaction of the drug with DNA isolated from *V. cholerae* cells was studied. (See figure 1 for a schematic summary). The drug was found to bind reversibly with DNA *in vitro* by intercalation between the base pairs[40]. Furazolidone binding inhibited significantly the thermal strand separation of DNA and also made it less susceptible to the action of DNase. This reversible binding of furazolidone with DNA *in-vitro* and also its fluorescence at 490 nm, when excited at 367 nm, led to the detection of the photobiological activity of the drug[90,91], a property till then unknown in the relevant literature.

The intercalative binding of the drug with DNA might resolve the issue of inhibition of DNA biosynthesis provided such a binding mode is found operative *in vivo* also, which however was not the case. It was soon revealed that the drug, immediately after its incorporation into the cell, underwent metabolic transformation and then bound to DNA. DNA isolated from drug treated cells was found to contain covalent bridges or inter-strand cross-links[87,92] as the drug-induced lesions. The diagnostic test revealing the presence of such covalent cross-links in DNA was that such DNA became reversibly bihelical[93]. This property of DNA isolated from furazolidone treated *V. cholerae* cells was identified and con-

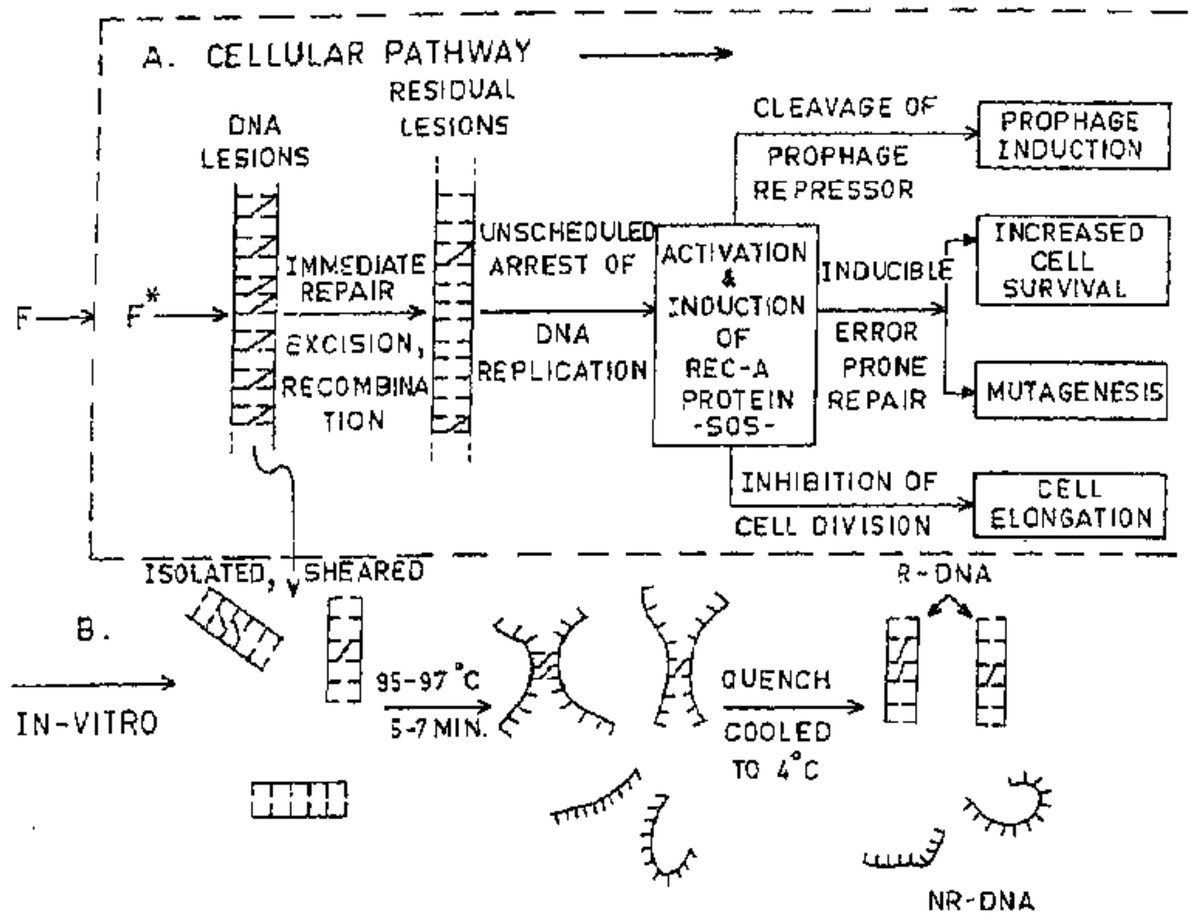


Figure 1. A. Sequence of cellular events following furazolidone treatment of actively growing *V. cholerae* cells as envisaged from the results of investigations in the author's laboratory. Furazolidone (F) is metabolically activated after incorporation into the cell. The activated derivative (F*) produces DNA lesions (inter-strand cross-links), most of which are removed by immediate repair processes. The residual lesions cause unscheduled arrest of DNA replication. This leads to induction of SOS functions, synthesis of more Rec A protein and activation of Rec A protein to become a protein-cleaving enzyme. Various forms of the protein are involved in three processes that give rise to four observed phenomena shown at right. Most of the important stages or phenomena of the cellular pathway have been experimentally detected but knowledge of details at some stages are yet to be documented (see text).
B. Sequence of events occurring when the *V. cholerae* DNA bearing furazolidone induced lesions are isolated, sheared and subjected to denaturation by short heat treatment. Quench-cooling of the denatured forms produces two types of DNA, one type regaining the bihelical form bearing the furazolidone-induced lesions and are termed reversible DNA (R-DNA) and the other type remaining as single stranded denatured forms having no drug-induced lesions and termed non-reversible or NR-DNA. The greater is the amount of R-DNA detected in this process, the larger is the number of lesions produced by the drug in DNA within the cell.

firmed by methods using UV absorption spectrophotometry, thermal chromatography through a hydroxyapatite column, fluorescence spectrophotometry of ethidium bromide bound DNA and SI endonuclease action. The reversibly bihelical DNA content increased with period of drug (0.5µg/ml) treatment and attained a plateau (80–84% reversibly bihelical DNA) after 45 min drug treatment at 37°C[87,94]. The initial phase of the furazolidone dose-response (formation of cross-linked DNA) relation was found to obey a first order reaction kinetics[94] indicating that the rate determining step of the reaction involved a single drug molecule. Assuming that the cross-links in DNA pool followed the Poisson Distribution Law, it was estimated that 80% reversibly bihelical DNA obtained after furazolidone treatment corresponded to about 161 cross-links per *V. cholerae* genome DNA[94,95]. DNA containing furazolidone-induced cross-links exhibited a melting temperature higher than that of native DNA and was significantly less susceptible to the action of DNase[87]. *V. cholerae* DNA bearing furazolidone induced inter-strand cross-links showed a change in the characteristic circular dichroism spectra of the DNA in dilute buffer[96]. The change in C.D. spectra was characterized by a shift of the positive band around 272 nm to lower wavelength, a loss of ellipticity of the negative band around 242 nm, was similar to that exhibited by mitomycin C-linked *V. cholerae* DNA under indentical conditions and was suggestive of a conformational change of DNA bearing such cross-links. Both furazolidone and mitomycin-induced cross-linking of *V. cholerae* DNA inhibited the salt induced conformation change, i.e., increase in winding angle of DNA, the percentage inhibition being greater for mitomycin-linked DNA.

5-nitro-2-furyl acrylic acid (5-NFA), another nitrofuran derivative, caused 80% of DNA in *E. coli* K-12 strain AB 2480 (*uvr⁻ rec⁻*) to become reversibly bihelical due to formation of inter-strand cross-links[97]. Nitrofurantoin, a urinary tract disinfectant, produced a maximal amount of 55% of reversibly bihelical DNA in *V. cholerae* OGAWA 154 by a drug dose of 120µg/ml × h indicating the formation

of inter-strand cross-links[98]. The same amount of drug could cause a maximum amount of 13.3% reversibly bihelical DNA in the repair proficient *E. coli* strain AB 1157. Again nitrofurantoin at the level of 3.0μg/ml × h only produced 65% reversibly bihelical DNA in the repair deficient *E. coli* strain AB 2480 (*uvr*⁻ *rec*⁻). This is comparable if not higher than that found in *V. cholerae* cells indicating that repair efficiency is the deciding factor in the net production and detection of inter-strand cross-links in the DNA of any cell. The difference in response of *V. cholerae* and *E. coli* cells to furazolidone action, as reported by McCalla[99], can be explained on this basis.

Recovery from furazolidone-induced DNA damage. Recovery of various cellular activities of *V. cholerae* subsequent to withdrawal of the drug presented an interesting pattern[87]. Withdrawal of the drug after 20 min pretreatment with 0.5μg/ml of furazolidone resulted in a faster recovery of DNA synthesis only. Complete recovery of the cells after pretreatment with 0.5μg/ml or 5.0μg/ml of the drug and its subsequent withdrawal was not attained within the next 3 h. On the other hand, *V. cholerae* cells held in buffer for more than 30 h subsequent to withdrawal of the drug significantly recovered from drug-induced damage[87]. Cells divided normally and regained their native size. DNA isolated from recovered cells lost the reversibly bihelical property, was susceptible, like native DNA, to the action of DNase and exhibited a melting temperature similar to that of native DNA.

An idea of the repair mechanisms involved in the removal of furazolidone-induced DNA lesions can be had from a study of the sensitivities of the different repair deficient mutant strains of bacteria. Since well characterized mutant strains of *V. cholerae* were not available, this study was done using the available and well characterized mutant strains of *E. coli*[94]. The double mutant *E. coli* strain AB 2480 (*uvr*⁻*rec*⁻) was by far most sensitive to the lethal action of the drug. The D_{37} (37% survival dose) values for the strains AB 2480 (*uvr*⁻ *rec*⁻), AB 2463 (*rec*⁻), AB 1886 (*uvr*⁻), AB 1157 (repair proficient) and AB 4410 (wild type) were 0.005, 0.015, 0.038, 4.0 and 4.0μg/ml × h respectively, indicating the relative importance of the different repair mechanisms in removing furazolidone induced DNA lesions. 'Rec-test'[100] was also applied to get statistically meaningful results in a shorter time. These results indicated the involvement of both recombination and excision repair mechanisms in the recovery from furazolidone induced damage[94].

Furazolidone action on *V. cholerae* is radiomimetic. The nature of DNA lesions induced by furazolidone on *V. cholerae* cells indicated that the drug action would be radiomimetic. Mitomycin-C, another cross-linking agent, also exhibited radiomimetic property[101]. Although the radiomimetic property of some nitrofurans, nitrofurazone in particular, was reported earlier, a quantitative relation between the doses of UV light (D_{UV}) and furazolidone (D_f) required for 10% survival of *V. cholerae* cells was reported by Banerjee and Chatterjee[102] as

$$D_f = 0.28 \times \text{Exp}\ (0.008\ D_{UV}).$$

Caffeine exhibited lethal synergism with furazolidone and the synergistic effect depended on the mode of caffeine treatment, the effect being maximum when caffeine was present along with and also after the furazolidone treatment.

Banerjee and Chatterjee[102] also made an interesting observation that the different vibrio biotypes presented distinctive patterns in respect of their sensitivities to the action of the drug furazolidone, *V. parahaemolyticus* strains being the most resistant and *V. cholerae* (classical) the most sensitive to this drug. The quantitative study should be of relevance to the clinical use of furazolidone in cholera cases. The radiomimetic property of furazolidone as revealed from studies on the strains belonging to different vibrio biotypes suggested that the enzymatic background for inducing lesions in the cellular DNA may be similarly effected at least partially by the radiomimetic agent (furazolidone) and the radiation concerned (UV light). However, the actual DNA lesions produced by these two agents are different.

Prophage induction by furazolidone. Prophage induction is produced after activation and induction of Rec A protein or by induction of the 'SOS' repair pathway[103]. Since well characterized lysogenic strains of *V. cholerae* were not available, prophage induction by furazolidone was investigated using the *E. coli* K12 strain GY 5027: *env A*, *uvr B*, *amp Al*, *Str A* (λ) as the tester and *E. coli* strain GY 4015: *amp A 601* as the λ-indicator strain[94,104,105]. By using the method 'Inductest III', as devised by Moreau and Devoret[106], a dose dependent prophage induction by furazolidone exhibited a gradual rise to a maximum, corresponding to an exposure dose of 50 ng/assay and a gradual fall thereafter at higher drug concentrations[105]. At least a two fold higher induction was achieved by using a metabolising mixture (liver extract) along with furazolidone. Chloramphenicol inhibited the furazolidone induced prophage induction indicating the need for concomitant protein synthesis for prophage induction to occur in the treated lysogens[105]. Several other nitrofurans were also reported to cause prophage induction[97, 98, 107].

Mutation of *V. cholerae* cells by furazolidone. Mutagenic activity of nitrofurans was reviewed by a

number of authors[99,107,108]. Furazolidone was also shown to induce reverse mutation in bacteria and forward mutation of *V. cholerae* cells from streptomycin-sensitivity to streptomycin-resistance[94,109]. The furazolidone induced mutation frequency increased with drug concentration, attained a maximum at the drug concentration of 7.0μg/ml and then declined with further increase of drug concentration. It is believed that the decrease in mutation frequency at doses higher than a certain value is the result of the lethal action of such agents at higher doses. The 5-nitro group is a basic requirement for mutagenicity and carcinogenicity of nitrofurans[99] since analogues without this group are essentially lacking in biological activity. The drug was also reported to induce reverse mutation, *his*$^-$ to *his*$^+$ revertants, in *S. typhimurium*[110] and *trp*$^-$ to *trp*$^+$ revertants in *E. coli* systems[107].

Induction of SOS repair by furazolidone. Furazolidone was shown (i) to induce filamentation of treated cells, (ii) to induce forward and also reverse mutation in bacterial cells and also (iii) to cause prophage induction in *E. coli* cells. The drug thus produces all the phenomena which are known to be produced after activation and induction of Rec A protein[103] or induction of 'SOS' repair pathway.

Is furazolidone carcinogenic? Furazolidone is one of the few nitrofurans which have found effective application in human medicine, particularly in the treatment of cholera, and hence extensive studies on its DNA damaging, mutagenic and carcinogenic activities are considered relevant and important. Whether furazolidone is carcinogenic or not has not been answered directly. However, the nature of the DNA damage caused by the drug, the involvement of the error-prone repair mechanisms and particularly 'SOS' repair in the removal of drug induced DNA lesions are enough indications to sound a note of caution about the possible carcinogenic activity. This concern is all the more significant since the drug has been reported to be mutagenic and to induce prophage in bacterial systems, mutagenicity and prophage inducing ability being considered as two of the recognised short term laboratory tests used for screening of carcinogens[103]. Under the circumstances, intensive investigation on the carcinogenic activity of this and related drugs of use in human medicine should be undertaken on animal systems.

It has to be admitted at the same time that important gaps in our knowledge on the molecular mode of action of the drug still remain. It has been generally recognised that furazolidone and related nitrofurans undergo metabolic activation immediately after being incorporated into the cells and then the activated derivative binds with DNA. While activation is usually considered to be catalysed by two distinct kinds of nitroreductases[105] (type I and II), further insight is needed into the details of the mode of activation and particularly differences in this aspect, if any, between the bacterial and animal cell systems. Also we do not know the nature or the chemical structure of the adducts formed in DNA. However, in spite of these inadequacies in knowledge, results of the investigations carried out in our laboratory and elsewhere are enough to warrant exercise of caution in the use of furazolidone in the treatment of cholera or other human diseases.

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ACKNOWLEDGEMENTS. The author expresses his sincere thanks to M/s Smith Kline and French (India) Ltd. for the gift of a sample of chemically pure furazolidone. He also thanks his former and present predoctoral students, J. Das, C. Raychaudhuri, P. C. Adhikary, Pratima Sur, M. Maiti, P. K. Chanda, Sujata Ghosh, S. K. Banerjee, A. K. Pal, Jayasri Basak, U. Mukherjee, D. Mandal, S. Rahman and Sukla Sengupta who participated at one or other stage during the progress of this research project on cholera.