Special Issue on S. N. De and
Cholera Enterotoxin

Cholera Toxin: From Discovery to Molecular Mechanism

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I vividly remember the visit that Professor S. N. De made to my parents' house just outside Oxford in 1976. It was a moving experience for my father, who had done some important pioneering work on the action of the cholera toxin that De discovered, and also for me, as I was working on the protein chemistry of the same toxin. It was a remarkable experience for us to meet a man who founded our field with one brilliant experiment.

My father, Kits van Heyningen (his real forenames were William Edward, but he was always known as Kits), died towards the end of last year, and so was not able to write a contribution to *Current Science* as I know he would have liked. He was a pioneer in the modern biochemical approach to the study of bacterial toxins. His book on that subject published in 1950 was the first for many years.[1] He had started as a result of work on wound infection during the Second World War and worked for example on the toxins of *Clostridium histolyticum* and of *Cl. welchii*, as well as with Shiga toxin.

However he was always anxious to work on the really big problems, and in the mid-1950s the outstanding toxin was that of tetanus: it is one of the most toxic of all proteins and causes a disease that kills millions, yet although it had been known and studied since the last century, essentially nothing was known of its mechanism of action. About the only piece of biochemical evidence likely to be relevant was the observation by Wassermann and Takaki published in 1898, that the toxin bound irreversibly to brain tissue. Kits van Heyningen's experiments were directed to showing what it was in brain tissue that actually "fixed" the toxin, and he soon found that it was two particular ganglioside molecules that bound to the toxin very strongly.[2] At that time, very little was known about the chemistry or biochemistry of the gangliosides, but they are now known to be a class of glycolipid found in all tissues, although in particularly high concentrations in brain. Since then many others, including me, have followed up this work, but there remains some doubt as to the true function of this binding to ganglioside. There is good evidence for something additional on the surface of cells that is also needed. We still know next to nothing about the molecular action of the toxin itself.

Cholera was always just as interesting a disease as tetanus, but at that time could not be studied biochemically, because there was no known toxin! When De's results showing that there was a toxin that could act in the absence of bacteria were published, the field opened up. My father did not really hear of this work until he went late in 1967 on sabbatical at Jack Craig's laboratory in Brooklyn, New York. At that point he became enthused with the value and importance of cholera research, and paid more than one visit to Bangladesh, observing patients and seeing for himself what a devastating effect cholera can have.

He became interested in using the toxin to make a vaccine, but it was several years before he started to do any biochemical work on it back in Oxford. Since studying fixation by tissues had proved so valuable with tetanus toxin, he began to do similar experiments with cholera toxin. Sure enough, gut scrapings bound toxin strongly, whereas many other tissues had no effect at all. Following the advice of his technician, presumably that anything is worth trying, he next tested the brain tissue which bound tetanus toxin so strongly – an experiment that looks like an irrelevant control since cholera toxin *in vivo* has no opportunity to get to the brain. Astonishingly, brain tissue fixed cholera toxin better than any other tissue. That made him think again of ganglioside, and he quickly showed that cholera toxin bound tightly and with high enzyme-like specificity to one particu-

lar ganglioside, known as GM1.[3] Subsequently this discovery has been remade by a remarkable number of people; I still get papers to referee in which the whole thing is stated yet again.

The discovery of the fixation of cholera toxin by ganglioside GM1 was the high point of my father's contribution to the field, and marked the start of a time of extraordinarily rapid movement in the investigation of the toxin, so that today it is one of the best understood of all toxins. Gangliosides are also among the best characterized of all biological receptors; their role as the actual toxin-binding component in cells has been demonstrated very clearly by many experimental methods, but principally by taking advantage of the fact that a cell without ganglioside GM1 in its membrane can take up the ganglioside from a solution *in vitro*, and thus be rendered susceptible to toxin. The end of my father's work in this field (and indeed of all his scientific work) was marked by the publication, in 1982, of his book *"Cholera: the American Scientific Experience 1947–1980"*, which he wrote together with Dr John Seal, and which gives a detailed account of the clinical, bacteriological and biochemical work.[4]

The experiments showing the binding specificity of cholera toxin were soon followed by others. I was looking around for a project once I had got a proper University job, when my father pointed out to me in 1972 that the protein chemistry of cholera toxin needed work that I might be able to do. Fundamental information about the structure of the toxin had already come from the laboratory in Texas of Richard Finkelstein, who was the first to purify the protein in large amounts, and, just as important for the progress of science, to make it available to those who wanted it. I was able to show quite easily, using the then relatively new technology of SDS polyacrylamide gel electrophoresis, that the toxin was similar to diphtheria and other toxins in that it had two different types of subunits, called A and B. It was the B subunits that bound to ganglioside.

At the same time, work starting in Michael Field's laboratory had shown that the toxin is a hormone analogue, activating the adenylate cyclase of eukaryotic cells. This made us think that the A subunit would perhaps have the direct effect on adenylate cyclase inside the cells, the binding of the B subunits having got it across the formidable barrier of the cell membrane. With my father's postdoc, Carolyn King, I was able to show that this was true. Several other groups were reaching similar conclusions at the time, showing that the A subunit catalysed the ADP-ribosylation of one of the regulatory G proteins of adenylate cyclase. (For general references to this work, see reference 5.) This very important group of proteins was essentially discovered through work with cholera toxin (and, later, with pertussis toxin).

When De published his paper, most of the work on bacterial toxins was done by scientists who thought of themselves primarily as bacteriologists or had a particular interest in toxins. Since then the toxins have become well known to many who know little of the disease or the bacteria, but are primarily interested in the biology of eukaryotic cells. I am sure most of those who have used cholera toxin to study the action of G proteins or the mechanism by which proteins enter cells, have little, if any, idea of what cholera is, know nothing of its bacteriology or pathology, and, I'm afraid, have never heard of De.

Several other toxins, such as diphtheria and pertussis toxins, and the C2 toxin of *Clostridium botulinum* also catalyse ADP-ribosylation. It is extraordinary that this reaction, which is probably a control mechanism in the normal physiology of cells, is also the mechanism of action of so many different toxins, produced by entirely different bacteria, and causing diseases that have nothing else in common except their great clinical importance.

What have we learnt from all that has been discovered about cholera toxin since De's discovery put in on the scene? One important idea, true of cholera toxin and of many others, is that toxins can, in principle, work in many more types of cells than they ever affect *in vivo*. Cholera toxin is active in the gut as De showed, and that is where the *Vibrio cholerae* grow in a patient. But ganglioside GM1 is found in virtually all eukaryotic cells, and cholera toxin is active everywhere: in intestinal cells and in erythrocytes, in slime moulds and in archaebacteria. Much early work on the activation of cyclase was done using erythrocytes from turkey and pigeon which are easy to work with and which respond very well to toxin; De must have been surprised to see that his toxin, whose activity he demonstrated in a relatively complicated intestinal system, actually worked everywhere once one knew what to look for.

Cholera is not the only toxin that is so widely active; diphtheria toxin kills most cells and pertussis toxin also activates cyclase almost ubiquitously. Even tetanus toxin, long thought to be a quintessential neurotoxin, is an inhibitor of exocytosis whose specificity for nerve cells is probably due to the fact that only they have enough of the specific toxin-binding gangliosides. Potentially interesting work is now being done by many groups including our own on the action of the toxin in other cells, particularly in adrenal chromaffin cells.

The lack of specificity of cholera toxin is partly a function of the ubiquity of the receptor. Yet although ganglioside is what binds the toxin, it is not a recep-

tor in the true pharmacological sense that binding to it produces the response. All that the binding does is to allow entry into the cell of the active A subunit. Many experiments with different toxins including cholera show that the nature of the binding 'receptor' is not particularly important. Since a cell may bind a million molecules of toxin on its outer surface yet only a few need to be inside for the biological effect, the uptake process can be one that is so inefficient that it would be quite inadequate for most proteins.

It is remarkable how little work since De's time has been done on the action of the toxin in intestinal cells; it has not even been strictly proved that it is activation of adenylate cyclase that produces the diarrhoea, although any other theory would seem perverse. The reason, no doubt, is that intestinal cells are not easy to work with: this is partly because their asymmetry is intrinsic to their activity, yet very hard to preserve *in vitro*. I have myself been working in the last few years on what processes might lie between the activation of cyclase and the subsequent ion movements that lead to the diarrhoea; we find a correlation between the anti-diarrhoeal effect of some drugs and their effect on toxin-catalysed ADP-ribosylation and cyclase activation, and we also find some protein phosphorylation produced by the toxin. The effects are not straightforward, but our hope is that this work could lead to the rational design of more effective anti-diarrhoeal drugs.

In the long run every scientist in this field would hope that his work would be of some benefit to people actually suffering from the diseases. De's critically important experiments were the starting point for more interesting science than anyone could have imagined, but, unfortunately, I doubt if most of those at risk from cholera have benefited significantly.

1. van Heyningen, W. E., *Bacterial Toxins*, Blackwell Scientific Publications, Oxford, 1950.
2. van Heyningen, W. E., The fixation of tetanus toxin, strychnine, serotonin and other substances by ganglioside, *J. Gen. Microbiol.*, 1963, **31**, 375–387.
3. King, C. A. and van Heyningen, W. E., Deactivation of cholera toxin by a sialidase resistant monosialosyl ganglioside, *J. Infect. Dis.*, 1973, **127**, 639–647.
4. van Heyningen, W. E. and Seal, J. R., *Cholera: The American Scientific Experience 1947-1980*, Westview Press, Boulder, 1983.
5. van Heyningen, S., Cholera Toxin, *Biosci. Rep.*, 1982, **2**, 135–146.

CURRENT IDEAS OF THE MECHANISM OF CHOLERA TOXIN

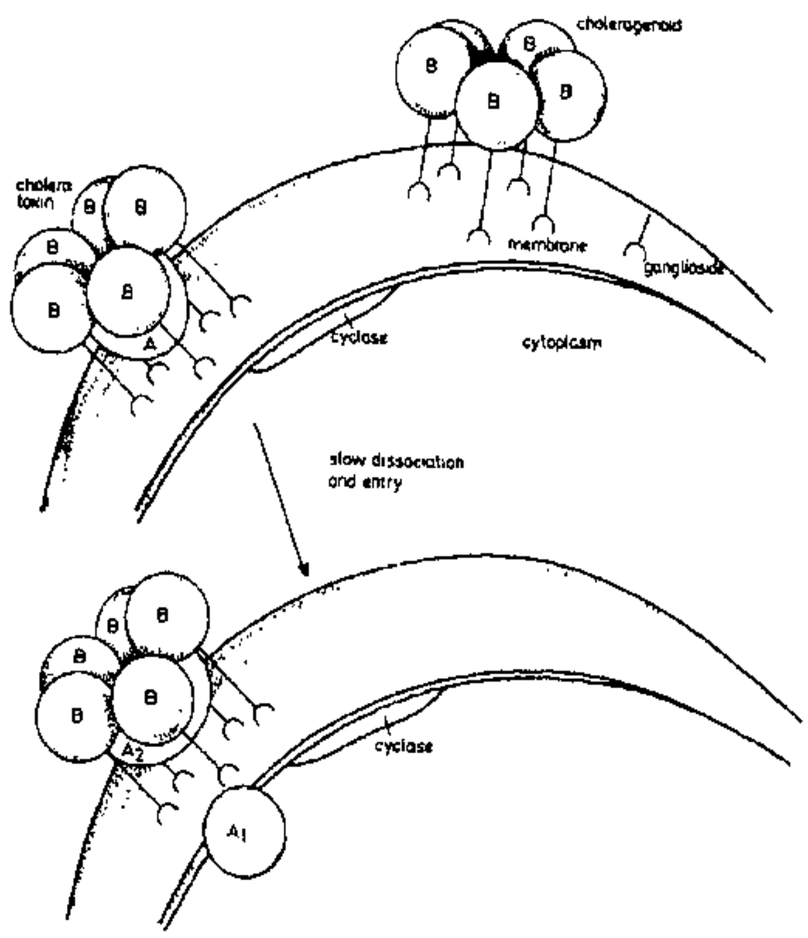

Figure 1. Entry of the toxin into the cell. A simple representation of the binding of intact toxin to the cell surface and the subsequent entry of the active A1 peptide. It is not certain that A1 actually separates completely from the rest of the protein, and, in intestinal cells, the situation is more complicated in that the catalytic subunits of adenylate cyclase are remote from the part of the cell where the toxin enters. Ganglioside molecules that are binding subunit B alone (choleragenoid) are no longer available for reaction with whole toxin. (Taken from S. van Heyningen *Cholera and Related Toxins*, in *Molecular Medicine* (A.D.B. Malcolm ed), IRL Press, Oxford, 1984. By permission of the Oxford University Press.)

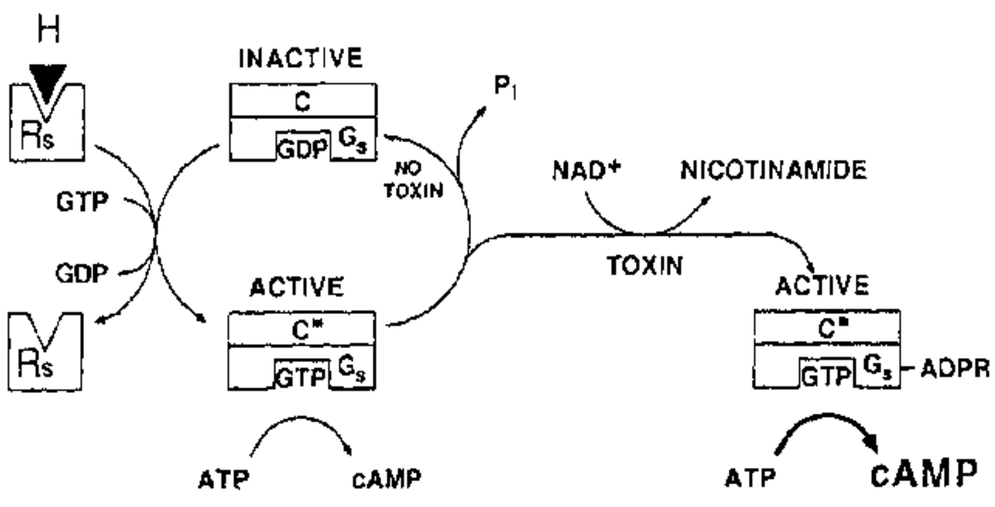

Figure 2. A simplified idea of the mechanism of activation of adenylate cyclase by the active A1 peptide of cholera toxin. H represents a hormone that binds to its receptor R_s; C and C* are inactive and active forms of the catalytic component of adenylate cyclase; G_s is the regulatory component: C is activated to C* only when G_s is binding GTP. The toxin catalyses ADP-ribosylation of G_s, preventing the hydrolysis of GTP to GDP, and keeping G_s in a permanently active conformation.