Special Issue on S. N. De and
Cholera Enterotoxin

The Berlin Cholera Conferences of 1884 and 1885*

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The first of the seven cholera pandemics (of which the last is still with us) erupted from India in 1817, but in its travel westwards got no further than Tiflis in Armenia and Astrakhan in Russia. The second reached Moscow in the autumn of 1830, Hamburg and then Sunderland in the north-east of England in October 1831, and New York in June 1832, killing 23 000 people in Great Britain and perhaps 2-6 times as many in the United States. A second wave in 1849 killed 53 000 people in Great Britain and twice as many in the United States. The third pandemic killed 23 000 people in Great Britain in 1853-4, but did not reach the United States. The fourth killed 14 000 people in Great Britain in 1866 and 50 000 in the United States. The fifth, in 1881, caused much less havoc in Europe than those that preceded it, but is notable for the fact that it was the one studied in the summer of 1883 by the French and German cholera commissions under Isadore Straus and Robert Koch in Egypt, and later by Koch in Calcutta.

Robert Koch (1843 – 1910)

As soon as cholera appeared in Europe, an understanding of its nature began to grow. In Moscow in 1830, two German expatriates in the Institute for Artificial Mineral Waters - Hermann, a chemist, and Jaenischen, a physician - recognized that all the water in the copious diarrhoeal stools (1.5 l/h) of cholera patients was derived from the blood, and could not be replaced because the ability of the small intestine to absorb ingested water was totally blocked. They suggested intravenous rehydration, but only one abortive attempt was made. When the cholera came to Sunderland two years later, William Brooke O'Shaughnessy, a 22-year-old physician qualified only in the previous year, saw straight into the heart of the matter. He analysed the blood and stools of cholera patients and concluded that they had lost not only the water from their blood, but also its neutral salts and the sodium bicarbonate. He suggested intravenous rehydration and remineralization with a solution that was then successfully applied in the rescue of a terminal case by Thomas Latta. But in the end the patient was lost, because nobody realized at that time that the process had to be continued until the infecting germ had been eliminated in the natural course of events. That was understandable, because the germ theory of disease was not established until 29 years later.

The germ theory of cholera came to the surface with the next pandemic which started in 1852. John Snow, Queen Victoria's anaesthetist, showed that the cholera 'poison' consisted of particles that were carried from one person to another by being swallowed, and then increasing in the alimentary tract. He

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proved with marvellous precision that these particles were carried by water contaminated by sewage, and suggested appropriate counter-measures. At the same time his Italian contemporary, Filippo Pacini, observed myriads of curved organisms swarming in the intestinal contents of cholera patients, and ascribed the cause of the disease to them, giving them the name *Vibrio cholerae*. So by the time Koch set off to Egypt to study cholera, everything he was going to learn there was already known, and published.

Koch reported and discussed the findings of his commission at the two sittings of 'Die Conferenz zur Erörterung der Cholerafrage' on 26 and 29 July 1884 in the 'Kaiserlichen Deutschen Gesundheitsamt' in Berlin; and at the 'Zweite Serie der Conferenzen zur Erörterung der Cholerafrage' on 4 May 1885. Although he was 'by common consent the greatest pure bacteriologist', as William Bulloch states, Koch committed two errors of omission and a grave blunder at those two conferences.

The first error of omission was to fail to acknowledge that the discovery that cholera is borne in water contaminated by sewage had been made by John Snow 30 years previously; the second was to fail to acknowledge that the causative organism had been recognized by Filippo Pacini 30 years previously. Perhaps Koch understandably preferred to find things out for himself, rather than burrow into the

William Edward van Heyningen, who was always known as Kits, was born in South Africa on 24 December 1911, one of seven children. His first degrees were in Chemistry and Geology at Stellenbosch University, and in 1934 he moved to England where he remained the rest of his life. His Ph.D. in Biochemistry was taken at Cambridge, and was followed by several years in the United States. He returned to Cambridge just before the outbreak of the Second World War, but moved to the Wellcome Research Laboratories in London in 1943, where he started his work on bacterial toxins. In 1947 he moved to the Sir William Dunn School of Pathology at Oxford University where he spent the rest of his career, ending as Reader in Bacterial Chemistry.

Apart from his scientific work at Oxford, Kits van Heyningen became very interested in the life of the University in general, and this interest led to his accepting the Mastership of a new College for graduate students, St Cross, which he held from 1965 until his retirement in 1979. He had several other important positions in the University, in particular he was a curator of the Bodleian Library and a visitor of the Ashmolean Museum for many years.

In 1940, he married Ruth Treverton, herself also a biochemist, and they had two children. His interests in life were not only centred around science and the University; in retirement he published an autobiography "The Key to Lockjaw", and a translation into English of Pagnol's French novel "L'Eau des Collines". He died on 27 October 1989.

English and Italian scientific literature of the pre-germ era.

But Koch's blunder delayed the discovery of cholera exotoxin for two-thirds of a century, and since cholera is a pure exotoxinosis, like tetanus and diphtheria, that was a serious matter.

At the first conference, Koch ignored the outstanding symptom of cholera, the diarrhoea, and stated that the symptoms of cholera that were usually thought to be caused by thickening of the blood were in reality caused by a systemically acting poison that had a 'paralytic cardiovascular' effect on the circulatory system. At the second conference, in 1885, Koch restated his poison hypothesis, and suggested that the poison belonged to the group of ptomaines that had recently been postulated by Brieger, but which in reality do not exist. It was this suggestion of a systemically acting poison that diverted the search for a proper animal model for testing the local action of cholera toxin, which is confined to the epithelial cells of the small intestine. When fresh culture filtrates of the cholera vibrio were injected intravenously into animals by various workers, no effects were observed; but when filtrates of older cultures were injected, various ill-defined toxic effects were reported. This led to the discovery by Cantani of bacterial endotoxins in 1886, and these were then pursued for the next seven decades with apparently no concern about the fact that none of the toxic symptoms observed bore the slightest resemblance to the diarrhoeal symptoms of cholera.

At last, in 1959, realism broke through, and it is not surprising that this happened in Calcutta, where the nature of cholera was understood. S. N. De, at the Nilratan Sircar Medical College, injected cholera culture filtrate into ligated loops of rabbit small intestine, and observed that after a few hours these loops swelled up with a rice-watery fluid characteristic of cholera stools. His short letter to *Nature* (1959) 183, 1533, on 'Enterotoxicity of bacteria-free culture filtrate of *Vibrio cholerae*' is a classic of medical and biochemical history. In the end it led to the current view of how cholera toxin turns on adenylate cyclase, producing the Second Messenger, which stimulates the secretion of chloride and bicarbonate ions into the gut, and at the same time blocks the absorption of sodium and chloride ions from the gut; and so brings about the accumulation of fluid in the gut that leads to the diarrhoea of cholera.

For further information, readers may be interested in *Cholera: The American Scientific Experience 1947-1980* by W. E. van Heyningen and J. R. Seal, The Westwood Press, Boulder, CO, USA, 1983.

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The Road to Understanding

These experiments should finally have exorcised the endotoxic irrelevance that had haunted cholera research since Pfeiffer invoked it in 1892. Indeed, they eventually did so, but their point was not recognized immediately. Even De at this stage could not entirely shake off the endotoxin, or parenteral administration of toxin. He showed that the enterotoxin-containing preparations did not cause a significant fall in blood pressure when injected intravenously into cats, whereas endotoxin-containing preparations did cause a marked and persistent fall. From this he concluded that both exotoxin and endotoxin played a part in the pathogenesis of cholera. But the effect of neither exotoxin nor endotoxin, when administered parenterally, is of any relevance to cholera, because neither toxin acts beyond the wall of the gut in the disease. The fall in blood pressure in cholera is due to the decrease in blood volume that results from the exudation of blood ultrafiltrate into the lumen of the gut.

In his next paper, De made another important contribution to the question of cholera toxin. He showed that cholera exotoxin is not produced by every culture of the cholera vibrio. Conditions for toxin production are very exacting – it depends on the strain of the subculture, the nature of the organic components of the medium, the concentration of these components, the salts, pH, aeration of the medium, and the time and temperature of incubation of the culture. Considerations of this kind have long been well understood by exotoxicologists. They underline the fact of life that any strain of the cholera vibrio grown in any medium under any conditions may well not produce cholera exotoxin, and therefore that the chances of cholera researchers in the pre-De era ever actually having had cholera toxin in their hands were slim – not that that matters, because the bath water that might have contained the baby was thoroughly thrown out anyway, and attention concentrated on the irrelevant placental cells.

Excerpted from *Cholera: The American Scientific Experience, 1947–1980* by W. E. van Heyningen and J. R. Seal.

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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-