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LEADING ARTICLE

The Cephalosporins and Sir EDWARD ABRAHAM: Recollections about a Great Scientist and His Part in the Discovery of These Antibiotics

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Personal Memories of Oxford and EDWARD ABRAHAM

I had the great good fortune—that I did not fully appreciate at the time—of working as a post-doctoral Fellow for $3\frac{1}{2}$ years (between 1967 and 1971) at The Sir William Dunn School of Pathology at Oxford University, in the very laboratories where penicillin and the cephalosporins had been developed. At that time Sir EDWARD was just Dr EP ABRAHAM, Reader in Chemical Pathology. The Professor of Pathology and Head of the School at that time was HENRY HARRIS, following in the line of such famous names as DREYER and FLOREY.

Although it is of course impossible to imagine what it was like working on the cephalosporins between 1949 and 1959, the atmosphere would probably have been similar to that when I worked there. It was all very calm and unhurried, almost casual. The day started late by modern standards but continued into the evening, although on Saturdays one could go home at teatime. There was always a week's holiday at Easter and Christmas (other Public Holidays were ignored), and the School closed down for three weeks in August. The Oxford University of those days was thus a very different place from what it is now, and had changed very little over half a century. This can be illustrated by the following:

* ABRAHAM told me how he had returned to Oxford in 1939 having completed a three year post-doctoral Fellowship abroad. It happened that his former supervisor saw him in the street and said "Haven't seen you for a while; have you had a cold?"

* One of the Lecturers at the Dunn School, a man with a brilliant recent career, decided he had achieved his ambition. There was thus no need to do any more work except to fulfil his statutory duties to give 12 lectures each year and to live within 6 miles of the centre of Oxford. When once I offered to lend him my bicycle, he remarked that he always walked everywhere, otherwise he would get back too quickly.

* A few years after I had left Oxford, ABRAHAM did me the great honour of asking me to examine one his DPhil students. When the oral had been satisfactorily concluded I casually asked the candidate why he had worked for three years on a topic that appeared to have no practical use whatever. The facial expressions of those present made it quite clear that it was totally out of order to expect an Oxford man to have done anything that could be thought of as "applied science". I was never asked to be an examiner again.

When I joined ABRAHAM's unit, the only senior permanent member of staff was Dr GUY NEWTON, whose title was Senior Research Officer. There were three PhD students, working respectively on purifying β-lactamase II (the Zn-containing enzyme) from *Bacillus cereus*[1], isolation of the antibiotic bacilysin[2], and the biosynthesis of cephalosporin C by protoplasts[3]. ABRAHAM also usually played host to a visiting Fellow from overseas—guests during the 1960s included JOHN KASIK (mycobacterial β-lactamase[4]), LEE SABATH (*Pseudomonas aeruginosa* β-lactamase[5]) and VLADIMIR BETINA (the antibiotic cyanein[6]). ABRAHAM and NEWTON themselves were working on, amongst other things, the biosynthesis of cephalosporin C, chemical modification of the antibiotic actinonin and the molecular changes that occurred when the β-lactam ring of cephalosporins was broken, either

chemically or by the action of β-lactamases. I became involved in the latter project, and when it was solved[7,8] we moved on to study the comparative immunological properties of cephalosporins and penicillins[9].

ABRAHAM was a quiet, almost shy man, from whom I, also an introvert, often had difficulty in extracting the help and advice I, as a microbiologist, needed to take forward what turned out to be a complex piece of chemistry. This was my own fault, as I was in awe of the great man and reluctant to disturb him when he was shut away, as he often was, in his own little study, which was the only private room in the unit. When approached, he always had an answer to my particular problem of the moment. On the other hand, GUY NEWTON was instantly accessible, as he did not have a room of his own. He was always forthcoming and extremely helpful, going out of his way to give assistance to anyone who asked. His sudden premature death during the Christmas holidays of 1969 was a great shock as well as a serious loss to science.

What I found fascinating about ABRAHAM, besides the vital part he played in the penicillin team and his leading role in the discovery of the cephalosporins, was his ability to see so far ahead. The project he set me to work on very soon showed that the immediate product of aminolysis or enzymatic hydrolysis from a cephalosporin was short-lived, the "cephalosporoate" decaying in a few minutes to give fission products. ABRAHAM immediately realized that this instability could be of fundamental interest to immunologists, as the hapten created when a cephalosporin reacted with body protein would have only a short existence before it changed into a different determinant. JAMES GOWANS, Professor of Immunology at the Dunn School, was fascinated by the possible implications of the existence of an unstable hapten, and would undoubtedly have worked on this problem had he not moved shortly afterwards to an Administrative post. ABRAHAM also realized that the chemistry of the reaction we were studying would become much clearer if we applied the relatively new technique of nuclear magnetic resonance. This is how I came to observe the β-lactamase mediated hydrolysis of a cephalosporin in an NMR spectrometer, all reagents being fully deuterated, in collaboration with EVA RICHARDS, the wife of the inventor of the technique, REX RICHARDS.

ABRAHAM's training as a chemist gave him an insight into the biochemistry of micro-organisms that opened up entirely new horizons. However, this precise discipline also accounted for a refusal to allow the publication of any phenomena that could not be fully explained, notwithstanding the fact that the biological literature is full of observations without adequate explanations, that microbiologists find of particular value. Although respecting his opinion, I regard it as unfortunate; he and NEWTON must have made many fascinating observations that they could not explain at the time during the 10 years of cephalosporin development, and the non-recording of these must represent a loss to science. On a personal basis, I regret being allowed to publish neither my finding that a certain isoxazolyl penicillin was extremely haemolytic nor my modification in 1968 of the Microtiter apparatus to allow MICs to be determined (this technology was originally conceived and at that time was marketed solely for immunological purposes). ABRAHAM also strongly disapproved of putting the names of technical staff on papers. This arose from an incident when, a technician having been cited as an author, someone from another University inadvertently requested their services as a PhD examiner. ABRAHAM had found explaining the situation deeply embarrassing, and thus vowed never to let anything similar happen again.

ABRAHAM spoke nothing of his personal part in the development of penicillin. However, each summer he would take us all out for an afternoon's punting trip, and he would occasionally reminisce about those days. Two stories especially stick in my mind, both concerning NORMAN HEATLEY. When HEATLEY had to fly to the USA during the war to brief his American collaborators on the culture of *Penicillium chrysogenum*, he impregnated the inner seams of his jacket with fungal spores, so that in the event of his aeroplane being shot down there was a chance that the fungus could be recovered if his coat were found. Again, it is no secret that during the early days of the penicillin work relations between HEATLEY and CHAIN broke down completely. They refused even to be in the same laboratory unless it was absolutely necessary, and did not speak to each other at all. When communication between them was essential for the continuance of the project, ABRAHAM had to act as a go-between.

Many feel that HEATLEY's contribution has not been emphasized sufficiently. For example, his ingenuity in inventing and developing the plate diffusion assay[10] has had a permanent influence of the practice of medical microbiology. HEATLEY was an incredibly precise man; I recall watching him during a very mundane journal club taking copious notes with a mapping pen in tiny, amazingly neat writing on the inside of a used envelope that he had opened up. He perfected the mercury piston pipette, and once showed me a micropipette he had made from discarded scraps of glass and rubber tubing. He announced with great pride "this delivers precisely 1.32 μl". I did not ask him what he intended to use it for, but I dare say he

found a purpose for it.

ABRAHAM had signed the Official Secrets Act during the war when he was working on penicillin, and this had, among other things, a lasting effect on the way he gave or showed papers to his colleagues. Long after he stopped handling secrets he used to slide documents over a desk, usually face down, instead of handing them out openly. As he soon discovered that I found it quite easy to read script that is upside down, he could not help being instinctively slightly suspicious when I was on the other side of a table on which he had papers, however innocent their content was. He was highly amused once when a visitor from the USSR at the height of the Cold War flew into a rage when he caught sight of a document labelled Top Secret lying on a bench when he was being shown round the Laboratory, thinking this was a trap designed to discredit him. In fact, the document was many years old, and it was there purely by chance; all its contents had become common knowledge. Another encounter that ABRAHAM had with the USSR caused him slightly less pleasure: on a lecture tour he was told that royalties were due to him for the manufacture of cephalosporins in that country, but that the accumulated roubles could be spent only in the USSR. He remarked with a wry smile that all he could have bought was a balalaika.

How the Cephalosporins were Developed

BROTZU decided in 1945 to test the sea water from around a sewage outflow pipe close to the University of Cagliari in order to explain the apparent absence of cases of typhoid (endemic in Sardinia at that time) arising from bathing in the sea and eating shellfish harvested in the vicinity. Among the organisms he isolated was a filamentous fungus identified as *Cephalosporium acremonium* (now renamed *Acremonium chrysogenum*) that when grown in his laboratory inhibited several important Gram-positive and Gram-negative pathogens. BROTZU used crude culture filtrates from this fungus to treat localized infections such as abscesses and systemic infections such as typhoid and brucellosis. Having published his findings in the one and only number of a journal specifically created for this purpose[11], he sought advice as to how more could be found out about his discovery, as he was aware that facilities to do this did not exist locally. Through the good offices of a British Public Health doctor whom BROTZU had met after the war, a culture was sent to the Dunn School of Pathology at Oxford, where HOWARD FLOREY asked ABRAHAM and NEWTON to investigate its antibiotic properties.

C.acremonium proved to be a remarkably prolific fungus. The first antibacterial compounds to be isolated and identified, the cephalosporin P family, were hydrophobic and active against Gram-positive bacteria only. They proved to have a tetracyclic triterpene skeleton, related to fusidic acid, that was discovered subsequently, and helvolic acid, that had been previously reported from the Dunn School. The chemistry and properties of this class of antibiotics have been well summarised by GODTFREDSEN[12]. Cephalosporin P could probably have accounted for the activity against staphylococci and streptococci reported by BROTZU, but does not explain the action of the culture filtrates against Gram-negative organisms.

ABRAHAM and NEWTON next found a hydrophilic antibiotic that was labile to penicillinase and gave a characteristic penillic acid when acidified. It was first named cephalosporin N, as it was active against Gram-negative species, but this was changed to penicillin N when its chemical structure was elucidated[13]. The sidechain of penicillin N, derived from α-aminoadipic acid, is charged at physiological pH values, that explains why its properties are different from those of other biosynthetic penicillins (*e.g.* penicillins G, V, O, K and F), in which the sidechain is not ionized. Penicillin N was difficult to purify, but in the early to mid 1950s was regarded as a potentially extremely valuable antibiotic due to its activity against Gram-negative pathogens, many of which were resistant or had acquired resistance to the few antibacterial agents clinically available at that time. Penicillin N was given an approved name (adicillin) and a few clinical trials were done, but when ampicillin came on the market production was discontinued.

It was during the studies on penicillin N that the crucial experiment was carried out in which cephalosporin C was discovered, by a chance observation. ABRAHAM and NEWTON needed to know the precise molecular weight of penicillin N; as the antibiotic was only available as a crude solution containing many impurities, their strategy was to convert penicillin N into its isomeric penillic acid, that was easy to isolate and purify, and determine the molecular weight of the latter. This involved acidification of an impure antibiotic solution that had been concentrated, and separation of various components in the resultant mixture on an ion exchange column monitored by following the ultraviolet absorption of the fractions. After the desired product, the penillic acid of penicillin N, had been eluted the column was for some reason kept running, revealing a later peak[14]. This turned out to be cephalosporin C, that was present in a very small amount and that had too low an intrinsic antibacterial activity to have been recognized by

bioassay in the original culture filtrate. In due course ABRAHAM and NEWTON discovered that cephalosporin C differed markedly from penicillin N, being stable to penicillinase and to acid, not forming a penillic acid and absorbing ultraviolet light. Its most exciting property at that time (the mid 1950s) was its consistent activity against *S. aureus*. The latter species, in the form of the "hospital staph, phage type 80/81" was in those days widespread in hospitals all over the world and had become very difficult (in some cases impossible) to treat. FLOREY encouraged ABRAHAM's wish to switch emphasis from penicillin N to cephalosporin C, which now became the focal point of research. Further work was made easier by the isolation of a mutant of *C.acremonium* that produced larger amounts of cephalosporin C. In due course, after much painstaking labour, NEWTON and ABRAHAM deduced that cephalosporin C consisted of a fused dihydrothiazine/β-lactam nucleus with an α-aminoadipoyl sidechain at position 7 and an acetoxymethyl group at C-3[15]. The details of this structure were disputed at the time, doubts being expressed in particular that the conjugated double bond system present would have an absorption maximum at 260 nm.

By now it was 1959, and workers at Beecham Research Laboratories had created the semi-synthetic penicillins by chemical modification of the penicillin nucleus 6APA[16]. The latter was available either by removal of the 6-sidechain of a natural penicillin using the enzyme penicillin acylase or by growing *P. chrysogenum* in the absence of sidechain precursors. However, neither of these routes could be used to make the cephalosporin nucleus 7ACA, without which semisynthetic derivatives could not be made. ABRAHAM and NEWTON were thus compelled to use chemical methods in order to perform the tricky operation of removing the 7-sidechain from cephalosporin C while maintaining the integrity of the nucleus. This was successfully achieved, albeit in very small yield, by acid hydrolysis; the resulting traces of 7ACA were acylated on chromatography paper with phenylacetyl chloride to yield the highly microbiologically active cephalosporin analogue (cephaloram) of penicillin G[17]. Shortly afterwards, workers at Lilly Research Laboratories used nitrosyl chloride to remove the sidechain of cephalosporin C in 40% yield, and this figure has been improved to 90% by later developments. Finally, it was found possible to expand the 5-membered thiazolidine ring in penicillins, *via* a sulphoxide, to the 6-membered dihydrothiazine ring, leading to a cephalosporin directly without the need to isolate 7ACA[18]. Although ABRAHAM was not involved in this particular work, the reaction fits in neatly with his continuing interest in the biosynthesis of cephalosporins[19], as a crucial stage in this is the involvement of an "expandase" enzyme that converts penicillin N into deacetoxycephalosporin C, precisely analogous to the chemical reaction mentioned above.

Afterthoughts

ABRAHAM wrote in 1970[20]: "some of the problems which had to be faced in the development of the cephalosporins seemed at the time to be so formidable that one wondered whether their solution would be possible". It was entirely due to his enthusiasm, flair and industry, together with NEWTON's meticulous experimental technique and attention to detail, that the project was carried through successfully. In addition, as usual, in terms of luck, fortune favoured the brave.

It is nonetheless fascinating to speculate what might have happened had one of the numerous twists and turns gone another way. Would the cephalosporins ever have been discovered had, for example, BROTZU not gone fishing for fungi in the Mediterranean, or had he not met Dr BLYTH BROOKE, or had the experiment to isolate penicillin N's penillic acid been terminated as soon as this compound had been obtained?

Given the soil screening programmes that were being actively pursued up until the end of the 1970s, and bearing in mind that several genera of bacteria (*e.g. Streptomyces* spp., *Azotobacter* spp., *Lysobacter* spp., *Xanthomonas* spp., *Flavobacterium* spp.) as well as fungi produce cephem antibiotics (*e.g.* cephamycins and cephabacins as well as cephalosporins), it seems quite likely that cephalosporins would have been discovered eventually. However, even had this been the case, it is doubtful whether they would have reached the pre-eminent position they occupy today, for which we have to thank chiefly Sir EDWARD ABRAHAM.

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