LEADING ARTICLE

EDWARD P. ABRAHAM, Cell-free Systems and the Fungal

Biosynthesis of Beta-lactams

ARNOLD L. DEMAIN

Biology Department, Massachusetts Institute of Technology,
Cambridge, MA 02139, U.S.A.

"The development of the β-lactam antibiotics could not have been envisaged by FLEMING in 1929, or by FLOREY and CHAIN when they initiated a further study of penicillin 10 years later Even in 1940 FLEMING wrote, "Penicillin has not yet been tried in war surgery and it will not be tried until some chemist comes along and finds out what it is and if possible manufactures it." In the event it was the microbiologists who were largely responsible for transforming what at that time seemed to be an almost impossible project, the economic production of penicillin by fermentation on a large scale."

Sir EDWARD ABRAHAM, 1983

Today much is known about the biology of penicillin and cephalosporin production by fungi including the pathways, the biosynthetic enzymes including some crystal structures, the genes and their cloning, expression, sequencing and chromosomal locations, the regulation of the genes and enzymes and even some intelligent guesses about their evolutionary relationships. The key breakthrough that led to rapid progress in these areas was the subcellular work done by EDWARD P. ABRAHAM and his Oxford colleagues in the early 1970s. With his advice and encouragement, my laboratory was able to prepare reliably active soluble cell-free preparations which were instrumental in elucidation of the biosynthetic pathways in fungi (and also in bacteria) by laboratories throughout the world.

I began my studies on penicillin biosynthesis at the Merck penicillin factory in central Pennsylvania in 1954. Tracer work had just been published on the incorporation of the entire L-cysteine molecule and the carbon skeleton of valine into the hydrophobic penicillin G[1,2]. Furthermore, the sidechain was found to be formed from phenylacetate[3]. Using washed and starved resting cells, I was able to show that cystine stimulated and S-ethyl-DL-cysteine and α-methyl-DL-valine inhibited penicillin G production by *Penicillium chrysogenum* and that these inhibitions could be reversed by L-cystine and DL-valine respectively[4]. Furthermore, D-valine was shown to be an inhibitor of penicillin synthesis while L-valine stimulated, suggesting that the precursor of the D-valine moiety was L-valine.

I next tested the effect of all the other protein amino acids on synthesis by starved resting cells and made the unexpected observation that L-lysine was inhibitory[5]. The results appeared to explain previous results by BONNER[6] who found lysine auxotrophs to be poor penicillin producers; the added lysine (required for growth) apparently inhibited the process. The significance of the lysine effect was unknown. Also unknown was the mechanism by which cysteine and valine condensed in forming the penicillin nucleus or the stage at which phenylacetate was incorporated as sidechain. Of interest here was the discovery by KATO[7] of an unknown compound (thought to be the "penicillin nucleus") only in fermentations conducted in the absence of the sidechain

precursor, phenylacetate. In 1955, I was able to confirm the production of "KATO's compound" cited in DEMAIN[8] which was later identified as 6-aminopenicillanic acid (6APA)[9]. Little did we know at that time that the lysine effect was related to sidechain attachment. However, we soon found out that lysine inhibition of penicillin synthesis by resting cells could be reversed by α-aminoadipate and that α-aminoadipate also stimulated penicillin formation in the absence of added lysine[10]. We concluded that the relative concentration of lysine and α-aminoadipate is crucial to penicillin formation, that α-aminoadipate has a role in penicillin synthesis, and that lysine interferes with that role. The role became clearer when the report of ARNSTEIN[11] was considered. These workers had found that δ-(α-aminoadipyl)cysteinylvaline (ACV) was formed intracellularly during penicillin G biosynthesis. They speculated that ACV is cyclized to a penicillin containing an α-aminoadipate sidechain which would then exchange this sidechain with phenylacetate to produce penicillin G. Of great interest in this regard was the isolation by FLYNN[12] from mycelium of *P. chysogenum* of a hydrophilic penicillin containing an L-α-aminoadipate sidechain, which was named isopenicillin N. It thus appeared probable[13] that isopenicillin N was the intermediate which exchanges its sidechain with phenylacetate to generate penicillin G. Since L-α-aminoadipate was known to be an intermediate in L-lysine biosynthesis in fungi, we further hypothesized that L-lysine was causing a negative feedback effect early in its own biosynthesis, thus limiting formation of L-α-aminoadipate which in turn would decrease formation of ACV and penicillin G[10].

The above concepts dealing with lysine inhibition were proven correct by later work done at M.I.T. in the early 1970's by my student, PRAKASH MASUREKAR, and my post-doctoral associate, CORNELIUS FRIEDRICH. We showed that feedback inhibition was the main mechanism of lysine control[14]. We further demonstrated that an early-blocked lysine bradytroph (= leaky auxotroph) which excreted homocitrate (the first compound on the lysine biosynthetic pathway) was feedback inhibited in homocitrate formation by addition of L-lysine[15]. Accordingly, lysine regulatory mutants of *P. chrysogenum* which overproduced lysine, were found to be poor producers of penicillin[16]. Although homocitrate synthase in extracts was insensitive to lysine presumably due to desensitization during preparation[17], the site of *in vivo* lysine action was shown to be homocitrate synthase by the fact that lysine inhibition of starved resting cells was reversed by homocitrate as well as by the later members of the pathway, *i.e.*, α-ketoadipate and α-aminoadipate[18]. We also found that the rate of penicillin

formation correlated directly with the intracellular concentration of α-aminoadipate[19].

A major breakthrough in β-lactam biosynthesis was provided by the discoveries made by EDWARD ABRAHAM on hydrophilic β-lactams in the 1950s and 1960s. GOTTSHALL[20] had discovered "synnematin B" in broths from *Cephalosporium salmosynematum* and CRAWFORD[21] reported on the production of "cephalosporin N" by *Cephalosporium* sp. ABRAHAM[22] found the compounds to be an identical penicillin possessing the hydrophilic D-α-aminoadipate sidechain. (Although the name "cephalosporin N" persisted for a few years, it was later more properly named penicillin N.) In 1955, a momentous discovery was made in the Abraham laboratory, *i.e.*, the isolation and identification of cephalosporin C from the penicillin N-producing *Cephalosporium* sp. culture[23]. Not only was cephalosporin C of intense interest because of its resistance to staphylococcal penicillinase, it also represented the first naturally-occuring β-lactam antibiotic without a 6APA nucleus, possessing instead the 7-aminocephalosporanic acid nucleus[24]. The culture also produced a steroidal antibiotic called "cephalosporin P"[25]. It was of interest that both penicillin N and cephalosporin C from *Cephalosporium* contained a D-α-aminoadipate sidechain whereas the isopenicillin N from *P. chrysogenum* contained an L-α-aminoadipate sidechain.

With regard to the biosynthetic pathway to cephalosporin C in *Cephalosporium acremonium* (known at various times as *Cephalosporum* sp., *Acremonium strictum* and, most recently, *Acremonium chrysogenum*), ABRAHAM's group showed that the acetoxy group is derived from acetate[26] and cysteine is incorporated intact into the β-lactam ring[27]. The sites of incorporation were the thiazolidine ring for valine and the sidechain for aminoadipate[26,27].

It was clear in the early 1970s that studies with intact cells had revealed the precursors of β-lactams but firm knowledge of the pathway and the enzymes involved was not at hand. What was needed was a new breakthrough involving cell-free systems and this was again provided by EDWARD ABRAHAM. At a symposium on antibiotics taking place in St. Marguerite in the Canadian Laurentians in early 1971, ABRAHAM announced that he and BRONWELL LODER had converted labelled phenylacetate plus coenzyme A and either 6APA or isopenicillin N to labelled penicillin using a broken cell system from *P. chrysogenum*[28]. He concluded that the reactions occurred in the following order: isopenicillin N → penicillin G ↔ 6APA. Also in that year, they[29] had isolated three peptides from mycelium of *C. acremonium*, one of which was δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (LLD-ACV). They postulated that

isopenicillin N was a precursor of penicillin N and cephalosporin C. ABRAHAM also reported that a broken cell system from C. acremonium could convert labelled valine plus δ-(L-α-aminoadipyl)-L-cysteine (LL-AC) to LLD-ACV, the activity being particulate in nature and not in the supernatant fluid. The presence of dipeptide LL-AC was essential for this reaction[30]; no labelled antibiotic was formed however. I had attended this lecture, and after returning from Canada, I wrote ABRAHAM and asked him to provide details on the preparation of the broken cell systems from P. chrysogenum and C. acremonium. On April 28, he and LODER kindly replied that the former was prepared by grinding with sand and the latter by ultrasonication, centrifugation and harvesting the particulate material.

The ability to produce tripeptide but not antibiotic from dipeptide plus valine using broken cells of C. acremonium[31,32] suggested to us that the enzymatic system responsible for the biosynthesis of β-lactam antibiotics was part of an unstable complex, and that the structure of this complex was destroyed by drastic treatments. In an earlier paper, ABRAHAM[33] had suggested that the conversion of cells into protoplasts followed by gentle lysis might preserve the integrity of the biosynthetic system. In preliminary work, DUNCAN and NEWTON[34] observed the incorporation of labelled L-valine into penicillin N by a sonicated protoplast preparation from C. acremonium. Furthermore, lyzed protoplasts could convert labelled valine in the presence of δ-(L-α-aminoadipyl)-L-cysteine, ATP and an energy-generating system into a penicillin; it also converted labelled LLD-ACV into penicillin N[32]. In a letter to me dated December 17, 1973, ABRAHAM stated that his lyzed protoplast-derived cell-free systems "may not be generally applicable, however, because we were only successful with one out of three other strains of P. chrysogenum". He further wrote that the yields of penicillin N produced from labelled valine by lyzed protoplasts of C. acremonium "are very low.... We badly need to increase the efficiency of this system and if we are successful I shall let you know. But until it has been improved I should have great hesitation in recommending it to you as a going concern."

Despite ABRAHAM's reservations, we thought the protoplast lysate approach was very promising[35] and that confirmation of the activity of the system was important. We prepared protoplasts of C. acremonium with enzyme preparations from either Helix pomatia (snail) digestive juice or Cytophaga. Protoplast yield depended on the enzyme used, the age of the culture and increased if the mycelium was pretreated with dithiothreitol. The cell-free

preparation, obtained by osmotic lysis of the protoplasts, synthesized labelled penicillin N from labeled valine but did not form cephalosporin C nor its precursors, deacetylcephalosporin C and deacetoxycephalosporin C. We had thus confirmed the cell-free studies of the Abraham laboratory. We next attempted to detect the synthesis of antibiotics by microbiological assay, rather than by using labelled precursor. We found that protoplast lysates yielded activity against a β-lactam-supersensitive mutant of Escherichia coli when incubated with an energy-generating system, mannitol, $MgSO_4$, KCl and Tris-buffer in short time experiments (up to 5 hours)[36]. The system was insensitive to Millipore filtration or cycloheximide and the product was a cephalosporin. The reaction was markedly stimulated by the addition of penicillin N but not by penicillin G or 6APA. This strongly indicated that penicillin N was being converted to a cephalosporin, the first time that biological ring expansion of a penicillin had been observed. Up until this time, penicillin N and cephalosporin C had been generally considered to be end products of a branched pathway in C. acremonium. Here it was demonstrated that penicillin N was a precursor of a cephalosporin. We went on to show[37] that the product made from penicillin N was deacetoxycephalosporin C (DOAC). Mutants blocked early in biosynthesis, which made no antibiotic in fermentations, were able to carry out cell-free ring expansion. Later blocked mutants, which produced penicillin N but not cephalosporin C in fermentation, were incapable of cell-free ring expansion and were presumably blocked in the deacetoxycephalosporin C synthase reaction. We gave the trival name "expandase" to this enzyme.

At this point, we were concerned that in both our laboratory[35] and that of ABRAHAM[32,34,38], lyzed C. acremonium protoplasts converted labelled valine into what was thought to be penicillin N but not into a cephalosporin. If it truly was penicillin N, why was it not converted into DOAC? We approached this problem by postulating that the product was not penicillin N but isopenicillin N, which had previously been detected only in P. chrysogenum. TOSHIO KONOMI, a visiting scientist from Japan in my laboratory, added LLD-ACV to our C. acremonium cell-free extracts and examined the antibacterial spectrum of the penicillinase-labile product. He had previously determined that penicillin N inhibited the growth of Salmonella typhimurium ATCC 13311 and Pseudomonas aeruginosa Pss but not that of Staphylococcus aureus ATCC 25923 or Sarcina lutea ATTC 9341, whereas isopenicillin N had the opposite pattern. We found the reaction product to have the antibacterial spectrum of isopenicillin N. At this time, we were preparing to present this exciting result at an

Fig. 1. Conference on "Mechanisms of Beta-Lactam Biosynthesis" organized by Professor DAVID PERLMAN (front row, extreme left) at the School of Pharmacy of the University of Wisconsin on December 15, 1978.

Sir EDWARD ABRAHAM is in front row center and the author is seated next to him (third from the right).
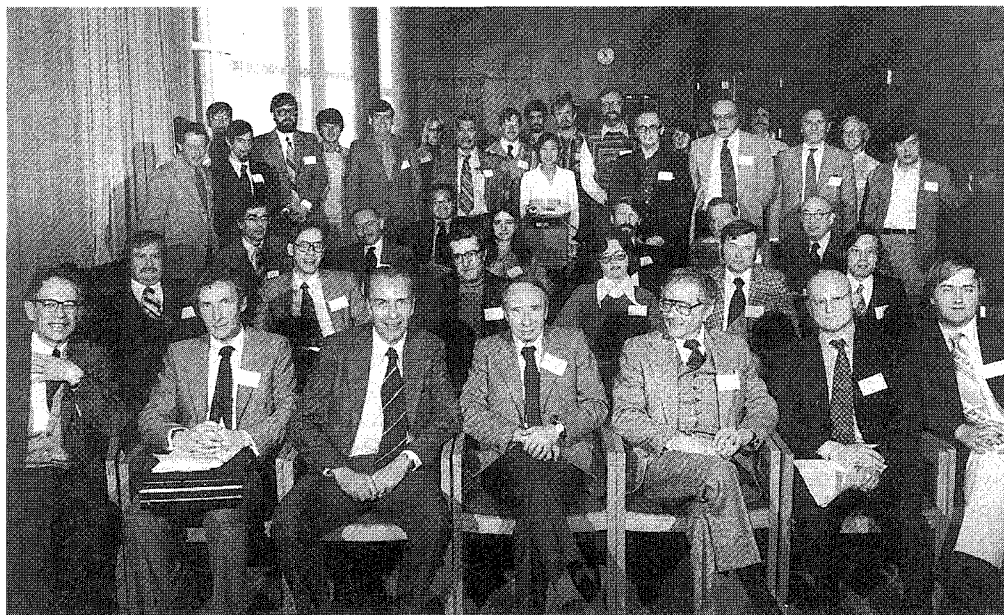
upcoming one day international symposium organized by Professor DAVID PERLMAN at the University of Wisconsin in honor of the cell-free breakthrough in β-lactam biosynthesis. We headed to Madison, Wisconsin, laden with our bioassay plates to demonstrate our discovery. Fig. 1 shows the distinguished international group gathered for the symposium. Professor ABRAHAM was given the initial spot on the program and I was to follow. I was shocked to hear him announce that O'SULLIVAN and he had made the same discovery by chemical detection of isopenicillin N. In discussing the findings of both laboratories with ABRAHAM after the meeting, he generously proposed that they delay publication of their paper until we prepared our manuscript. As a result, both papers[39,40] appeared back-to-back in the same issue of the Biochemical Journal.

Cell-free extracts were also crucial in defining the role of 6APA, or lack of one, in penicillin G biosynthesis. Although 6APA accumulated in *P. chrysogenum* broths to which no sidechain precursor had been added, we doubted that free 6APA had a role in *de novo* biosynthesis of penicillin G[41]. We felt that it was merely a shunt metabolite derived by deacylation of isopenicillin N in the absence of phenylacetate addition. Cell-free studies had indicated that the terminal reaction of penicillin G biosynthesis was an exchange of the L-α-aminoadipate sidechain of isopenicillin N for phenylacetate from phenylacetyl-coenzyme A. Such acyltransferase activity had been found in crude extracts and phenylacetyl-CoA had been shown to arise from phenylacetate and coenzyme A by the action of a sidechain activating enzyme. The appearance of the enzyme preceded rapid penicillin G formation and declined at the same time as the penicillin G production rate in fermentations. In the absence of phenylacetyl-CoA, isopenicillin N was hydrolyzed to 6APA by penicillin acyltransferase[42]. This free 6APA may be converted later to penicillin G if phenylacetyl-CoA becomes available. This reasoning implicated isopenicillin N as the true immediate precursor of penicillin G and 6APA acylation as a salvage pathway. The observation in the Abraham laboratory[43] that extracts could convert isopenicillin N, but not penicillin N, to penicillin G was consistent with the above concept.

Rapid progress was made following the cell-free discoveries described above. The ring expansion of penicillin N to DOAC was improved markedly by the work of HOOK[44], our own later studies[45,46] and those of FELIX[47], which showed the importance of ascorbate, α-ketoglutarate

and iron for this dioxygenase reaction. The preceeding reaction, the cyclization of LLD-ACV to isopenicillin N (to which we gave the trivial name "cyclase", otherwise known as isopenicillin N synthase or IPNS), was found to be stimulated by ferrous ions[48]. For preparing cell-free extracts containing active cyclase and expandase, the laborious protoplast formation and lysis procedure was replaced by simple sonic oscillation.

The question still remained as to why the cell-free *C. acremonium* preparations converted LLD-ACV or valine into isopenicillin N but not into penicillin N and cephalosporins. The answer was provided by KONOMI[40] who showed that fresh cell-free extracts could convert LLD-ACV to a cephalosporin but frozen and thawed extracts could not, the reaction sequence stopping at isopenicillin N. Thus an extremely labile epimerase [called "racemase" by KONOMI[40]] existed which could convert isopenicillin N to penicillin N, thus changing the configuration of the α-aminoadipyl sidechain from L to D. The existence of this epimerase and its extreme lability were later confirmed in Oxford[49,50].

It is quite remarkable that within two years of the KONOMI's[40] paper, a scheme of penicillin and cephalosporin biosynthesis, including the steps of cephamycin synthesis by actinomycetes, could be presented[51] and remains essentially correct today. The further hydroxylation of DAOC to deacetylcephalosporin C was shown to be catalyzed by cell-free extracts containing another α-ketoglutarate-linked dioxygenase[52~54]. The conversion of deacetylcephalosporin C to cephalosporin C was found to be carried out by the enzyme acetyl CoA: deacetylcephalosporin C acetyltransferase[52,55].

Once the active cell-free preparations were available, purification of the cyclase, epimerase and expandase proceeded in our laboratory[46,56] and in others[57~59]. However, up until the mid-1980s, the reaction(s) leading to LLD-ACV was still unknown. It appeared the dipeptide LL-AC must be involved because of the early finding by[30] that broken cells could incorporate ^{14}C-DL-valine into LLD-ACV but only when LL-AC was added. GERALD BANKO in my laboratory spent a full year trying to demonstrate the production of LL-AC from L-α-aminoadipate plus L-cysteine by cell-free extracts of *C. acremonium* but without success until he used 50% glycerol during sonication of the cells. With this modification, he was able to carry out a linear synthesis of LL-AC from its constituent amino acids for at least 6 hours, the reaction being dependent upon ATP and Mn^{2+} or Mg^{2+} [60]. Obviously we were dealing with a very labile enzyme activity. Although at that time, we thought LL-AC to be

a free intermediate, we later found that it was really an enzyme-bound intermediate of the ACV synthetase reaction[61]. We reasoned that if two enzymes were required for LLD-ACV synthesis from the component amino acids and the first exhibited inhibition by its product, LLD-ACV formation *via* the reaction LL-AC + L-valine would be faster than LL-AC formation from L-α-aminoadipate + L-cysteine. We found this to be the case; however we unexpectedly observed that the conversion of the three amino acids to LLD-ACV was far more rapid than either of the two half reactions. We thus concluded that our cell-free preparation contained a single enzyme (which we designated ACV synthetase) which required the simultaneous binding of all three amino acids for maximum activity. We were able to support this conclusion by purification of ACV synthetase from *C. acremonium*[62]. A summary of this work on ACV synthetase has been published[63] as well as the use of this enzyme as a model system for enzymatic peptide synthesis[64].

Today, much is known about β-lactam biosynthesis[65]. It is clear that the sub-cellular enzyme work begun in the Oxford laboratory of EDWARD P. ABRAHAM provided the breakthrough needed by the field for elucidation of the pathways leading to fungal penicillins and cephalosporins. It also opened the way for our understanding of the production of cephamycins, clavams and other β-lactams by the actinomycetes and other bacteria. The isolation of the pure enzymes, determination of their crystal structures[66], discovery of the genes involved[67] and their chromosomal locations, elucidation of the regulation of the genes and enzymes[68,69], cloning and expression of the genes in heterologous hosts[70] and consideration of the evolutionary relationships[71] all followed on the heels of the development of reliable cell-free systems. I will always be indebted to the late EDWARD P. ABRAHAM for his scholarship, his highly ethical character and his generous offers to help a young industrial microbiologist contribute to the world of technology and science.

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