Papers and Originals

Origin and Function of Penicillinase: a Problem in Biochemical Evolution*

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Brit. med. J., 1967, 4, 71–77

The title of this lecture is intentionally provocative. It is well known that there are many varieties of the enzyme that can split the β-lactam bond of penicillins and/or cephalosporins (Fig. 1)—the specific reaction referred to generally as "penicillinase." And their physiological role in the life history of the bacteria which produce them is still a matter of controversy. But the case I wish to argue will be one in favour both of recognizing the relation between the various "brands" of the enzyme, despite many striking chemical differences, and of considering the possibility of a common or similar evolutionary origin.

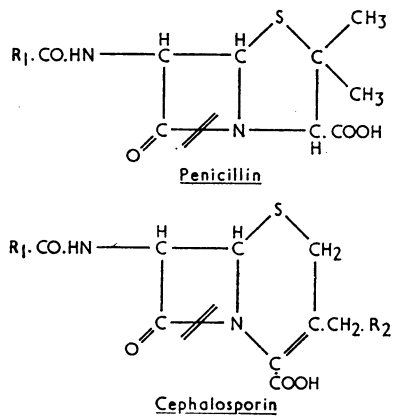

FIG. 1.—Basic structure of penicillins and cephalosporins, showing the position of the main variant side-chains (R_1, R_2) and the specific β-lactam (CO–N) bond (indicated by the oblique double line) that is split by "penicillinase."

At our present state of knowledge the history of penicillinase began in 1689. I know there was no enzymology in those days, but I'll explain how a little later on.

The penicillin and cephalosporin β-lactamases are produced by a wide range of Gram-positive and Gram-negative species of bacteria, and differ from each other enormously in the relative rates at which they hydrolyse different substrates. The range of substrates, which differ (in the penicillin class) in the side-chain (R_1) attached to the free NH_2 group of the "nucleus," 6-amino-penicillanic acid, and (in the cephalosporin class) in that side-chain and also in the side-chain (R_2) attached to C_3 of the cephalosporanic acid nucleus, runs into many thousands. Most types of the enzyme, though predominantly attacking either the cephalosporins or the penicillins, will hydrolyse some members of the other group of compounds at a significant rate. It is the Gram-positive species that produce enzyme with the highest activity and also in greatest quantity. This, when the relevant gene is maximally active (fully "derepressed"), may amount to about 2% of the total bacterial dry weight, or probably over 3% of its protein.

In most of these strains the system is inducible: the penicillinase gene is usually strongly repressed, and production of the enzyme is minimal until a penicillin or cephalosporin is added to the culture, after which it may increase several hundredfold.

In most instances the enzymes are certainly responsible (probably exclusively) for the high degree of resistance to penicillins and/or cephalosporins manifested by the organisms that produce them. (For recent accounts of their biological, chemical, and physicochemical properties see the reviews by Pollock (1965a) and Citri and Pollock (1966).)

"Function"

The penicillinases of Gram-positive bacteria are among some of the most active enzymes known: second only to catalase. Their specific activities, expressed as turnover numbers (molecules of substrate split by one molecule of enzyme per minute at 30° C.), range around 1.5×10^5 (Pollock, 1965b).

Nevertheless, there are certain difficulties in accepting what many believe to be the naive assumption that the main or sole function of penicillinase, in the orbit of the organisms that produce it, is to destroy penicillin.

The objections raised can be classified under four main headings. I will deal with them systematically and try to produce the counter arguments as we go along.

1. Substrate Specificity

It has been claimed that the enzyme may have a broader specificity than that provided by the β-lactam bond of penicillins and cephalosporins (Saz and Lowery, 1964; Saz *et al.*, 1964). The a priori argument is that the penicillin nucleus is really only a condensation of two amino-acids, L-cysteine and D-valine, with an additional cross-bridge, and that the CO–N bond split is only a modified peptide link (Fig. 2). Penicillinase, then, might be regarded as a sort of modified peptidase: its penicillin-hydrolysing ability could be "incidental."

Original suggestions (Saz *et al.*, 1964) that certain peptides were in fact hydrolysed by what were claimed to be purified preparations of *Bacillus cereus* penicillinase have not, however, yet been substantiated or successfully repeated.

Rather more impressive were reports from these authors of the specific inducing activity of certain straight chain and

* Being the substance of the first Almroth Wright Lecture, in the 1967 series, given at the Wright-Fleming Institute of Microbiology, London W.2, on 1 May 1967.

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cyclic oligopeptides, on wild-type inducible penicillinase systems in *Staphylococcus aureus* and *B. cereus*. Their findings have not yet been consistently reproducible in all laboratories where they have been investigated. But the idea behind them is a particularly interesting one, and only a few weeks ago further reports appeared from Saz's laboratory (A. K. Saz, personal communication) claiming that induction of *B. cereus* penicillinase had been demonstrated with extracts from the cell walls of *Staph. aureus* containing muramic acid and peptides. The suggestion here, again, is that penicillinase is really a modified peptidase, perhaps one originally mainly concerned

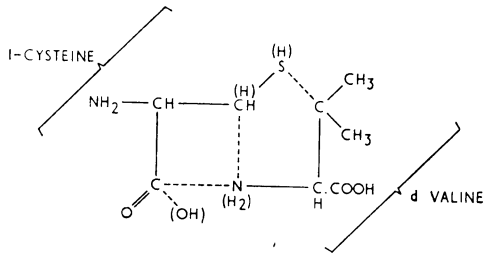

FIG. 2.—Basic structure of penicillin viewed as a cyclic dipeptide: L-cysteinyl-D-valine.

in cell-wall metabolism; but in any case not originally or primarily orientated towards the β-lactam ring of penicillins and cephalosporins.

We shall consider the possible relation of the enzyme to cell walls, from an evolutionary point of view, later on. But at the moment it is essential to point out that:

(*a*) No isolated and identified substance other than penicillins and cephalosporins has ever been unequivocally shown to act as substrate for purified preparations of β-lactamase—that is, so far as possible uncontaminated with other enzymes. Even if some (slight) peptidase activity had then been demonstrated it would hardly alter the significance of the overwhelming orientation of purified enzyme towards this particular β-lactam bond.

(*b*) Strains of *Staph. aureus, B. cereus,* and *B. licheniformis* genetically deprived of their β-lactamase activity—be it through a point mutation in the penicillinase gene or a complete deletion by plasmid elimination from the cell—show a profound loss in resistance to the penicillins. As might be expected by reason of its extracellular location and detoxifying action, the enzyme is a collective weapon against the antibiotic and does not often, in these organisms, give much protection to single isolated cells. But if resistance is tested with whole populations the effect of penicillinase can be shown to increase their penicillin-resistance by several orders of magnitude—for example, from 0.01 unit to several hundred units/ml. in *Staph. aureus,* and from 0.01 unit to several thousand units/ml. in *B. cereus* and *B. licheniformis* (M. R. Pollock, unpublished observations).

2. Significance of Low Penicillinase Activities

Many (fully induced) Gram-positive bacteria produce very high levels of activity—5,000 units per mg. dry weight being not at all unusual. However, among Gram-negatives—in particular the coliforms—activities may be so low (<1 unit per mg. dry weight) that it is difficult, at first sight, to believe that it could be of any value to the cells as an anti-penicillin agent. Moreover, there is often rather little apparent correlation between penicillin and cephalosporin resistance among coliforms and levels of their β-lactamase, as measured in the laboratory.

There are, however, a number of factors operative in this sort of comparison which make an assessment of function extremely difficult:

(*a*) Strains differ considerably in their "intrinsic" susceptibility to penicillins and cephalosporins. This, possibly, is a question of the sensitivity or accessibility of cell-wall synthesizing enzyme systems to these antibiotics; at all events it has nothing directly to do with penicillinase. Many penicillinase-producing coliforms have a high intrinsic resistance, and the extra margin of resistance contributed by β-lactamase might be expected to be, for that reason, smaller than for those organisms with low intrinsic resistance.

(*b*) It may be most misleading to translate enzymic activities, as usually measured in the laboratory, into enzymic function in vivo under natural conditions. There are here three main relevant factors to be taken into consideration, as follows:

(i) The chemical and physical cell relationships and extracellular environment may be very greatly and significantly different in nature—for example, in the soil or in animal and plant tissues—from those normally operative in laboratory assays, where enzyme solutions or homogeneous cell suspensions have normally to be used. The degree of cell clumping, the local concentrations, and "feed-in" supply of substrate, etc., may be expected to be particularly important in relation to detoxifying enzymes.

(ii) The location of the enzyme with respect to the cell may be of vital significance. There may be advantages for an intrinsically sensitive cell to produce a penicillinase that is extracellular (like *B. cereus, B. licheniformis,* and some strains of *Staph. aureus*) for "clearing a way free of penicillin" in order to prevent the antibiotic actually reaching the susceptible cell itself (Pollock, 1962). But this involves a huge dilution effect, so that large quantities of the enzyme would need to be produced (as indeed appears to be the case with exo-penicillinase-forming organisms).

With different types of cell it may be more effective to have very small amounts of enzyme strategically located next to the cell-wall synthesizing enzyme systems on the surface of the cell—or attached to the membrane, as might indeed be the case with many of the coliforms which have small quantities of enzyme firmly and completely bound to cell structure (Smith, 1963a; Smith and Hamilton-Miller, 1963; Hamilton-Miller, 1963). It would be of great interest to know whether this enzyme is concentrated at "growing points" on the cell surface (in so far as they may be restricted in number and location), where active synthesis of cell-wall material is occurring—see reviews by Salton (1964) and Rogers (1965).

(iii) Enzymic efficiency in vivo can probably only rarely be gauged by measurement of maximal activity ("Vmax") in enzyme assays. This is because, in the latter, the enzyme is, by definition, always saturated with its substrate, whereas there are good reasons for believing that in the intact cell most metabolites are at very low concentrations, and their enzymes are therefore highly unsaturated. Operational enzyme activity is determined as much by affinity for the substrate as by turnover number. This means that in vivo the rate of the reaction catalysed by one unit of any given enzyme is governed as much by the concentration of its substrate as by its maximum specific activity.

These considerations apply especially to the penicillinases, where the affinity constant or "Km" (defined as the substrate concentration at which the enzyme functions at 50% maximal efficiency), when measured for the common penicillins, is nearly always well above the minimal growth inhibitory concentration of the antibiotic.

A more valid measurement for any particular variety of the enzyme would therefore be one which has been referred to as "physiological efficiency," being defined as Vmax/Km (Pollock, 1965b). This is directly proportional to the rate of substrate hydrolysis under conditions of gross enzyme undersaturation, and allows direct comparison between different penicillinases at any given penicillin concentration. By using this value it has been possible to show that varieties of penicillinase from the same species may differ at least sixfold in Vmax but not significantly at all in physiological efficiency. The reason for this can be interpreted quite simply in enzymological terms; but the essential point here is that there is no difference between the rate at which they hydrolyse penicillin at the low concentrations normally expected in nature.

These factors still, of course, do not take into account further discrepancies that may arise between enzymic measurements on soluble extracts (which are necessary for controlled enzymological work) and those made in vivo with enzyme that is bound to intact cells. With whole cells, substrate-accessibility barriers (Smith, 1963b; Smith and Hamilton-Miller, 1963; Sabath *et al.*, 1965; Ayliffe, 1965; Hennessey, 1967) and/or factors altering the shape or "conformation" of the enzyme molecule associated with its binding to other

structures may often be operative and affect what might be termed the "cellular efficiency" of an enzyme.

(*c*) In certain instances normal penicillin-sensitive wild-type strains of bacteria may be found to produce measurable but exceedingly minute quantities of penicillinase (Ayliffe, 1963, 1965; Smith and Hamilton-Miller, 1963; Eriksson-Grennberg *et al.*, 1965). It would seem at first sight to be an utterly useless activity. However, in some instances cells mutate spontaneously to form greatly increased quantities of the identical type of penicillinase. They thereby acquire greatly increased penicillin-resistance (Pollock, 1957; Eriksson-Grennberg *et al.*, 1965).

The classical case is that of a variety of *B. cereus* studied by Sneath (1955), where overnight culture of the original penicillin-sensitive strain in broth containing 1 unit of benzylpenicillin/ml. permitted its conversion to a new ("mutant") population of cells forming the same enzyme at 5,000 times the previous rate, with 300 times the resistance to the antibiotic—even when tested against single cells. The phenomenon was unequivocally shown to be due to a spontaneous mutation (presumably in some type of regulatory gene), the effect being to cause complete derepression of a pre-existing penicillinase structural gene, followed by selective overgrowth of the mutant by the penicillin present.

Previously, such combined mutation-plus-selection phenomena in bacteria were termed by Stanier (1953) "evolutionary adaptation" to distinguish them from "physiological adaptation," which included enzymic adaptation processes involving all the cells in a population and no change in genotype.

I do not, however, believe that this particular phenomenon has anything at all to do with evolution in the general sense. Rather it would seem to be a mechanism of biological adjustment, itself evolved as a type of populational adaptation to meet the needs of a whole community of individuals in the face of an intermittently intruding situation. Such a situation—the presence of toxic quantities of penicillin—may be supposed to have been previously encountered often enough to have allowed the acquisition of a fully fashioned structural gene for an enzyme which, however, is kept rigidly repressed (for economy of effort), apart from occasional "release" mutants which can be immediately selected by the penicillin if and when it presents itself in the environment.

3. Formation of Penicillins and Cephalosporins in Nature

So far, attempts to demonstrate the formation of these antibiotics in soil under natural conditions have not been successful, and a certain scepticism has developed about whether they are even indeed capable of being produced except as laboratory freaks (see Brian, 1957).

There are, however, several reasons why it might be difficult to isolate them from the soil: they are likely to be present in extremely low concentrations; they may be rapidly destroyed by the chemical environment or adsorbed on soil surfaces; and they may, indeed, be hydrolysed by the penicillinase formed by the large numbers of *B. licheniformis* and *B. cereus* cells undoubtedly present in most types of soil all over the world.

It does not really seem that attempts to demonstrate their formation have been anywhere near exhaustive.

Moreover, recently a strain of *Penicillium chysogenum* has been coaxed to form penicillin, by growth on the surface of sterilized soil—rather capriciously, admittedly—and will form the antibiotic more regularly, under strictly controlled conditions, in aqueous soil extracts containing only lactose as additional nutrient factor (P. Hill, private communication).

4. Physiological Role of Penicillins and Cephalosporins

It is also sometimes argued that even if these antibiotics are formed under natural conditions they can be nothing other than waste products which are fortuitously toxic to certain bacteria. It is further argued that, in any case, susceptible bacteria must meet the antibiotics only very rarely, and it is therefore unlikely they would have evolved enzymes specially to deal with them.

There are quite a number of points that must be emphasized here:

(*a*) Penicillins and/or cephalosporins are not rare oddities in the fungal world, as was once supposed. Table I lists no fewer than nine distinct genera of microfungi—comprising a total of at least 36 species (there are probably in reality many more)—that produce one or both of these two classes of antibiotic. All these genera may inhabit the soil at one time or another, and three out of nine are dermatophytes, normally saprophytic on skin and/or other keratinized body surfaces.

TABLE I.—*Microfungal Genera Forming Penicillins and/or Cephalosporins*

	Aspergillus (7 species) (see Sanders, 1949; Cole, 1966)
	Cephalosporium (see Abraham, 1962)
D	*Emericellopsis* (see Kavanagh *et al.*, 1958; Uri and Valu, 1963)
D	*Epidermophyton* (Uri and Valu, 1963; Cole, 1966)
	Malbranchea (see Sanders, 1949)
	Paecilomyces (Pisano *et al.*, 1961)
	Penicillium (23 species) (see Sanders, 1949; Cole, 1966)
	Streptomyces (Miller *et al.*, 1962)
D	*Trichophyton* (see Sanders, 1949; Uri and Valu, 1963; Smith and Marples, 1964; Cole, 1966)

D = Dermatophytes.

(*b*) All the main penicillinase-producing species of bacteria are either natural inhabitants of soil and water in large numbers (*B. cereus* and *B. licheniformis* and *Pseudomonas*) or are found there from time to time (such as *Aerobacter, Klebsiella*)—or they are typical skin saprophytes (*Staphylococcus*). In other words the natural environments of penicillin-formers seem to be similar to, if not identical with, those of penicillin-destroyers.

It is probable that strains of *Penicillium* or *Aspergillus* are not infrequently present together with *B. cereus, B. licheniformis,* or *Pseudomonas* in the same sample of soil (Holding *et al.*, 1965). And penicillinase-forming *Staph. aureus* has been found coexistent with penicillin-forming *Trichophyton* on the skins of hedgehogs in New Zealand (Smith and Marples, 1964).

There seems no need to puzzle why some strains of *Staph. aureus,* isolated in 1937 well before the therapeutic era of penicillin, were found to produce the same penicillinase in just as potentially large amounts as that formed by "modern" post-penicillin strains. Therapeutic penicillins have, of course, certainly enormously increased the *incidence* of penicillinase-positive strains of staphylococci—presumably by natural selection (see Ciba Foundation, 1962). But they have not detectably altered the nature of the penicillinase genotype (M. H. Richmond, private communication). It can be argued that the evolutionary stimulus had already been there previously—perhaps for many millions of years.

(*c*) Finally, it seems a priori probable that most soil organisms are engaged in a perpetual struggle for survival in an environment where supply of nutrients is a constant factor limiting growth and multiplication. Any weapon reducing the growth rate of a competitor might be expected to help survival and further growth of the organism producing it.

It is indeed possible to demonstrate, in the laboratory, that both *P. chrysogenum* and *B. licheniformis* inoculated together (the latter somewhat after the former) into the same soil sample will grow to a final total number which is considerably lower than that attained by either alone, when inoculated into an aliquot sample separately (M. R. Pollock, unpublished experiments).

The stage could therefore be said to be set for a battle between the microfungi and the bacteria, such as to encourage the evolution both of the penicillins and cephalosporins on the one hand and of the enzymes to hydrolyse them on the other.

Moreover, it must be remembered that penicillinase is known only among those organisms with *N*-acetylmuramic acid-containing mucopeptide in their cell walls (the synthesis of which is specifically inhibited by the penicillins) which are sensitive to, and threatened by, penicillin. If penicillin hydrolysis is only an incidental by-reaction of another type of enzyme activity with real physiological significance, why is it not found in organisms that do not have to bother about penicillin?

At least it can be said that the situation, as thus analysed, makes teleological sense.

The evidence is, however, circumstantial, and it must be accepted that this interpretation remains highly questionable. All that can be claimed is that it is not unreasonable.

"Origin"

1. Physiological Adaptation

(*a*) In general, most wild-type penicillinase-producing strains of bacteria isolated in the absence of penicillins and cephalosporins show very low enzymic activity. However, nearly all Gram-positive strains and a proportion of Gram-negatives are typically penicillinase-inducible, and respond rapidly to treatment with the antibiotic by increasing rates of enzyme formation up to 300 times the "basal" level. No permanent genetic change is involved, and the cultures revert to their usual uninduced level of production after a period of growth in the absence of inducer (see Pollock, 1959).

I do not propose to deal further with this well-known phenomenon of enzyme induction here. It is as vital a part of resistance to the antibiotics as direct enzymic action itself.

(*b*) As mentioned previously, there is another method by which wild-type "micro-constitutive" strains—both among Gram-negatives (Eriksson-Grennberg *et al.*, 1965) and Gram-positives (Sneath, 1955)—can increase their rate of penicillinase formation: what I have called a *populational adaptation*, involving an apparently spontaneous mutational event in a very small proportion of a population, derepressing the penicillinase structural gene to allow its full expression, followed by selection of such mutationally altered cells in the presence of a penicillin concentration which will suppress the growth of the rest of the population.

Although the change is genetically fairly stable, it will materialize rapidly, and there are grounds for regarding it as a physiological type of adaptation when applied to the whole population.

2. Genetic Control

(*a*) The populational adaptation referred to above can also, of course, in another context, be regarded as a genetic type of acquisition of increased penicillinase activity.

(*b*) More fashionable, and better studied at the moment, is the mechanism by which cells can acquire penicillinase activity—from scratch, so to speak—by acceptance of extrachromosomal factors (episomes or plasmids) which can carry the β-lactamase gene complex in a state that is partially autonomous from the chromosome, but otherwise functions in the coding and control of formation of the enzyme as do the chromosomal genes.

In coliforms the penicillinase genetic system may constitute part of an extrachromosomal "R" or resistance factor, which, by linkage up with a "T" or transfer factor, may pass from one cell to another spontaneously (not necessarily within the same species) in the process referred to as "infectious heredity" (Datta and Kontomichalou, 1965).

In *Staph. aureus*, "penicillinase" plasmids can be transferred from one cell to another—*not* (so far as is known) spontaneously, but by transduction through an infecting bacteriophage (see Richmond, 1965a; Novick, 1967). It is possible, with staphylococci, for the cell to possess more than one type of penicillinase plasmid at the same time. And in this organism it seems likely that the same or analogous penicillinase genes can, in certain strains, exist fully integrated on the chromosome (Asheshov, 1966), though there is yet no clear evidence that they can pass reversibly from the extrachromosomal to the chromosomal state like episomes.

3. Evolutionary Origin

When we come to consider the problem of evolution we enter the realms of high speculation. In spite of the fact that we accept the fact of organic evolution unquestioningly—at least I *suppose* we do—we know extremely little about its mechanism. Yet so many biologists seem tacitly to assume, rather uncritically, its neoDarwinian basis and its occurrence through a process of natural selection operating on random "spontaneous" mutations and recombinational events in the genome.

With the rise of molecular biology it is now possible to visualize possible steps at a chemical level and be a great deal more precise in our hypotheses.

Unfortunately we do not yet know the exact amino-acid sequence of any type of penicillinase—though such an analysis for one of the staphylococcal varieties is only just short of completion (R. P. Ambler, private communication). Nothing, of course, is known about the base sequences in the relevant deoxyribonucleic acid of its gene. So speculations must be linked to rather more general considerations of function and properties.

At the beginning of this lecture I emphasized the variety in properties of the different pencillinases, particularly in immunology, substrate profile (relative activities on various penicillins and cephalosporins), and overall amino-acid composition.

I now wish to do the reverse and point out the similarities which are (perhaps a little tendentiously) stressed in Table II (see also Pollock, 1967). The number of individual amino-acid residues may differ considerably, but the combined basic and combined acidic amino-acids are fairly similar; cysteine is absent; the specific activities range only over a factor of 10 (which is very narrow considering the wide range of values for enzymes in general) and the "physiological efficiencies" (previously defined) only fourfold.

TABLE II.—*Properties of Penicillinases from Different Bacterial Species*

	B. cereus	*B. licheniformis*	*Staph. aureus*	*E. coli*
No. of varieties examined	2	2	2	1
Molecular weight	31,000–35,000	28,000	29,000	16,700
N-terminal amino-acid	Asp.	Lys.	Lys.	
Amino-acid composition (No. of residues per molecule):				
Cysteine	0	0	0	
Lysine + arginine	40–43	38	48–49	
Aspartic + glutamine	60–69	64–66	57–64	
Total hydrophobic	67–85	75	66–75	
Differential rate of synthesis (μg. enzyme/mg. dry bacterial weight)	9–11	11–12	8–9	1·0
Molecular activity $\times 10^5$ (No. molecules benzylpenicillin hydrolysed/molecule enzyme/min. at 30° and pH 7)	1·5	0·2–1·1	0·2	0·19
Physiological efficiency $\times 10^6$ (for definition, see text)	5–6	5–6	16	4

From data summarized by Citri and Pollock (1966).

Though the amino-acid compositions of the *B. cereus* and *B. licheniformis* varieties are as different from each other as either is from the staphylococcal enzyme, there is a striking though quantitatively slender immunological cross-reaction between these two (Pollock, 1967), which would indicate some partial homology in tertiary structure and therefore probably elements of amino-acid sequence resemblances which may also occur in other types. There are, however, no similar peptides found on tryptic hydrolysis of the staphylococcal and *B. licheniformis* enzymes (R. J. Meadway, private communication).

The molecular weights of the varieties from Gram-positive organisms are almost identical, whereas that from *Escherichia coli* (Datta and Richmond, 1966) is about half that of the

others. There is good evidence that in all varieties there is only one N-terminal amino-acid residue, and therefore a single peptide chain, and it could be argued (as in the case of haemoglobin chains—Fitch, 1966) that the Gram-positive variety originated from an "internal" duplication of the more primitive Gram-negative chain.

There is perhaps significant evidence supporting some extent of common pathway in the evolutionary history of these enzymes. It seems unlikely that the degree of biological and structural similarity evidenced could have occurred from a purely convergent line of development.

Be that as it may, we shall assume for the moment that the penicillinases have had a common evolutionary origin, though it is not strictly necessary to do so for the argument I shall develop.

I shall also accept two other propositions, both of which are reasonable and in fashion—but very far from being proved:

(*a*) That the structural genes for most existing enzymes have evolved through modification of pre-existing structural genes for other enzymes which must inevitably be closely related in amino-acid sequence and therefore probably similar in biological specificity.

(*b*) That the most plausible mechanism for ensuring the stability of useless intermediary steps in the evolution of one enzyme from another during the period before a significantly useful version had emerged would be through preservation of the existing enzyme by an initial duplication of the relevant gene. In diploid organisms this might not be necessary, at least not at the first stage. In haploids it would be immediately essential and in diploids eventually so, except in so far as such evolution could be by substitution rather than by addition. The expansion of the genome through phylogeny must imply that most evolution has been through addition.

The mechanisms by which the "second copy" is modified need not really concern us here. It could be through genetic shuffling: by recombination, translocation, deletion, or inversion; but in most cases probably through a sequential series of point mutations.

In what direction do these two assumptions point?

Taking the latter first, it is implied that evolution might proceed faster in diploids because initial modifications to one allele would be automatically protected in their early "useless" stage, at least in heterozygotes, without having to wait for linear reduplication. Homozygotes, however, would become lethal and highly disadvantageous, and so duplication would tend to follow at some stage.

So far no bacterial species have been found to be completely diploid, but temporary partial diploids are frequent in staphylococci (Novick and Richmond, 1965; Novick, 1967)—either in the sense of two allelic extrachromosomal plasmids or plasmids plus chromosomal alleles. In that sense—which could, however, be relatively unimportant—the situation may have been favourable for the evolution of penicillinase, whose genes are now known to be capable of forming diploids—at least under laboratory conditions (Richmond, 1965b).

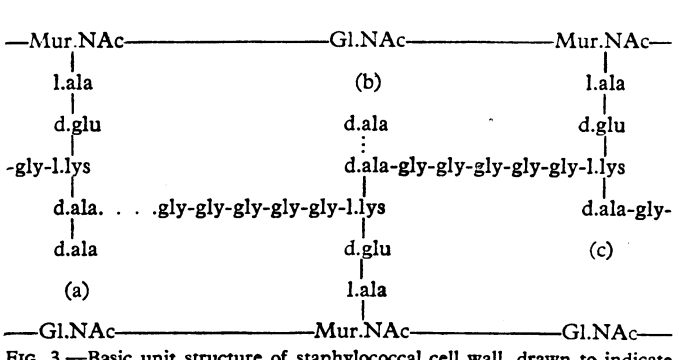

FIG. 3.—Basic unit structure of staphylococcal cell wall, drawn to indicate the two last steps (a and b) in the specific penicillin-sensitive transpeptidation reaction which completes the bridge (c) between the two different oligopeptide chains, and thus finally rigidifies the whole macromolecular complex into a stable molecular exoskeleton for the cell. Based on an article by Wise and Park (1965). Mur.NAc=*N*-acetylmuramic acid. Gl.NAc=*N*-acetyl glucosamine. Abbreviations for amino-acids as suggested by the *Biochemical Journal*, 1967, **102**, 3.

The first assumption implies that we should look for the evolutionary origin of penicillinase among enzymes attacking or combining with molecules resembling penicillin. And where better should this be done than among those dealing with the biosynthesis of the cell-wall mucopeptide, a process we know to be specifically inhibited by penicillin (see Park, 1966) and one of whose enzymes, therefore, is a likely candidate?

Fashions change in the interpretation of penicillin antibiosis. At the moment the reactions in cell-wall synthesis which have most attention in staphylococci are ones directly involving the exchange of the terminal D-alanine residue of the oligopeptide chain attached to muramic acid, with the first glycine of the pentaglycyl peptide attached to L-lysine of a near-by oligopeptide chain. This reaction, which may be one of the last of the sequence in cell-wall synthesis, thus forms a cross link between two such peptide chains (see Fig. 3). It has in fact been shown to be extremely sensitive to inhibition by penicillin in vitro (Wise and Park, 1965).

It has also been pointed out (Collins and Richmond, 1962) that *N*-acetylmuramic acid shows a strong three-dimensional structural analogy to penicillin (though superficially there is little resemblance between their chemical formulae). Tipper and Strominger (1965), however, stress rather the analogy with d-ala-d-ala, emphasizing that penicillin is a modified peptide, and that the penicillin-sensitive transpeptidation reaction itself is a type of peptidase (which, it has been argued, is also true of penicillinase). Ironically, Wise and Park (1965) and Park (1966) stress rather the L-alanyl-γ-D-glutamyl part of the cell-wall molecule as being most closely analogous to penicillin.

It has in fact been claimed in Saz's laboratory—as mentioned previously—that extracts of *B. cereus* cell wall can induce penicillinase in this organism. Induction tests are very sensitive, and they are easier to perform than tests for substrates. They can thus reasonably be taken, if properly controlled, to indicate the possibility of enzymic affinity for the substances as substrates; though it certainly does not necessarily follow that inducers are substrates—or indeed complexants of any kind—for the enzyme. (We have, however, tested *N*-acetylmuramic acid and the d-ala-d-ala dipeptide as inducers for the enzmye in *Staphylococcus* and *B. cereus* without finding a trace of activity.)

If the *N*-acetylmuramic acid/penicillin analogy is significant, another candidate for ancestry would be the extracellular bacterial lysozyme first described in *Bacillus subtilis* (Richmond, 1959) but probably also produced by many other species. Like the lysozyme from egg-white, this enzyme probably attacks the cell-wall mucopeptide by hydrolysing the *N*-acetylmuramic acid/*N*-acetyl glucosamine bond. It might therefore conceivably be structurally related to penicillinase.

Indeed, following the elucidation of the tertiary structure of egg-white lysozyme by Blake *et al.* (1965) it was reported by Johnson (1967) that this enzyme does indeed bind penicillin to a significant extent, at a site which also binds a derivative of *N*-acetylmuramic acid. Penicillin, moreover, was also found to inhibit the action of lysozyme. The groups on the molecule that appear to be involved in this binding reaction with lysozyme are not, however, apparently those originally thought to form the basis for the postulated homology with *N*-acetylmuramic acid (Collins, 1967). The significance of this reaction remains therefore uncertain.

Other interesting candidates would be: (*a*) the enzymes in lysostaphin (Schindler and Schuhardt, 1964, 1965) among which may be some peptidases responsible for splitting the d-ala-gly bond that is formed by the transpeptidase previously mentioned as being specifically inhibited by penicillin; and (*b*) the "competase" described by Young *et al.* (1964) which splits the link between *N*-acetylmuramic acid and the L-lys

residue that is the first of those making up the peptide "side-chain," and confers transformation competence on *B. subtilis*.

What of course is needed here is the isolation and characterization of these cell-wall enzymes in order to see if there are any amino-acid sequence homologies or other types of resemblances between them and the relevant penicillinase.

However, the fact remains that penicillin does inhibit a step or steps in cell-wall biosynthesis quite specifically, and this must almost inevitably mean that it combines specifically with one of the enzymes involved. Moreover, penicillin is known to combine with high affinity, and very specifically, with some sort of receptor site (the P.B.C. or "penicillin-binding-component"—see Cooper, 1956) which is present only in those organisms that are highly susceptible to penicillin. This component could logically be one of the cell-wall synthesizing enzymes, and it is not unreasonable to suppose it might have evolved, through modification of the site that combines with penicillin, to become capable of hydrolysing the antibiotic instead of simply being inhibited by it. This possibility, and related ones, have been discussed recently by Boman *et al.* (1967).

Evidence

In attempting to tackle the general problem of penicillinase evolution itself, is it possible to obtain any direct evidence of the mechanism?

We have made efforts along these lines of approach as follows:

(a) Palaeoenzymological Evidence

In a survey of a wide range of different present-day isolates of *B. licheniformis* from all over the world, strains could be sharply classified into two groups according to the properties of the penicillinase they produced (Pollock, 1965b). The enzyme occurred in two related but quite distinct molecular types, differing by only a very few (probably two or three) amino-acid residues and a number of enzymological properties.

Accordingly, when Sneath (1962) revived some spores of *B. licheniformis* which had lain dormant in the dried soil stuck to the roots of some plant specimens preserved untouched in the British Museum since 1689, the opportunity was seized of examining the properties of the penicillinase produced by the strains found (M. R. Pollock, unpublished experiments). They all fell clearly into one of the two types existing at present: their substrate profiles and immunological reactions indicating that no evolutionary change had occurred at least during the last 270-odd years—a short enough time, of course, in absolute terms; but in terms of the maximum possible number of cell generations, equivalent to 100 million years or so on the scale of human generation time, and therefore presumably long enough for evolution to have occurred if cell generations were the main operative factor.

(b) Laboratory Studies

"Blind training" of the existing penicillinase genome by serial subcultures of *B. licheniformis* in marginally inhibitory concentrations of the new penicillins, such as methicillin, did not give rise to cells producing penicillinase with increased ability to hydrolyse the compounds in question.

Attempts to follow sequential mutations in the penicillinase structural gene, isolated after mutagen treatment of *B. licheniformis*, that might have some physiological significance in changed enzyme specificity yielded nothing of interest. It was, however, possible to show that single-step, presumptive point mutations, supposedly involving single amino-acid residue substitutions in the molecule, could produce a complete inversion of relative penicillinase and cephalosporinase activities (M. R. Pollock, unpublished observations). In other words, a single-step mutation could result (in two instances: see Fig. 4) in an enzyme originally predominantly a penicillinase being converted into one that was predominantly a cephalosporinase. But in neither case was there a clearly significant absolute increase in specific activity. It could therefore hardly be claimed that the change was one that must have evolutionary significance. Nevertheless, it might be the first step in a significant evolutionary sequence, and these instances at least indicate the considerable alteration in substrate specificity that may result from a single mutation, and can be regarded as possible examples of the sort of event that may be occurring in the process.

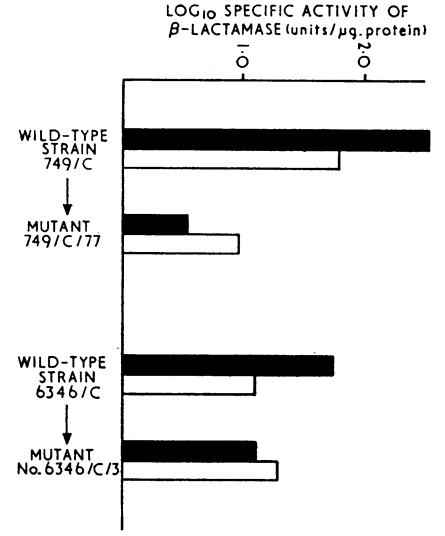

FIG. 4.—A mutational "conversion" of penicillinase into cephalosporinase. Comparisons of specific activities (units/μg. protein) in hydrolysing penicillin and cephalosporin of wild-type and mutated penicillinases produced by two different strains of *B. licheniformis*. ■ Penicillinase (benzylpenicillin as substrate). □ Cephalosporinase (benzyl ceph. C as substrate for 6346 strains and cephaloridine for 749 strains).

It is conceivable that the type of modification observed here is not far removed from the naturally developed change that may be presumed to have occurred in the past in the evolution of the two extracellular β-lactamases of *B. cereus* recently separated and characterized by Kuwabara and Abraham (1967). These two types (one of which is predominantly, if not exclusively, a penicillinase (β-lactamase-1: the original α-penicillinase) whereas the other has approximately equal activity on cephalosporins and penicillins (β-lactamase-2)) differ in thermostability, activation by Zn^{++} and other characters. Their comparative structural properties are so far unknown. But in their response to induction and the simultaneous conversion of the systems controlling their formation to constitutivity, by a single mutational step, they behave as if their genes were components of one operon—that is, contiguously located on the chromosome and under a single control system. They may therefore, for this reason alone, be considered to be possibly evolutionarily related.

(c) Field Survey

The advent, and therapeutic use on a wide scale, of the new penicillins, such as methicillin, cloxacillin, and oxacillin, which are very resistant to hydrolysis by staphylococcal penicillinase, raised the possibility that the organism might respond by developing a modified enzyme that would more effectively destroy these compounds. The prospect of following the evolution of a new enzyme with biochemical and genetical checks at every stage seemed an interesting possibility. Accordingly, a systematic biochemical survey of newly isolated strains of staphylococci showing increased resistance to methicillin was instituted at the Central Public Health Laboratory, Colindale.

The results so far have been unequivocally negative. Increased methicillin-resistance has undoubtedly occurred, and the number of isolates showing some rise in resistance to methicillin has increased. But no new type of enzyme has emerged, and there has been no significant increase in the quantity of enzyme produced (Richmond, 1966; K. G. H. Dyke, unpublished results).

The increase in resistance, which in any case has been modest, is of the "intrinsic" variety and could be due to a cell-wall

synthesizing machinery with a greater capacity for standing up to the penicillinase-resistant penicillins than that predominating in pre-methicillin days. Its biochemical nature and the mode of its emergence remain a mystery.

The most important single factor contributing to the development of penicillin resistance in staphylococci in recent years would appear to be probably the infectious spread of penicillinase plasmids mentioned previously.

Conclusion

All these scrappy and indecisive points, the indirect and circumstantial evidence, and the speculative and perhaps tendentious arguments may seem rather naive and primitive attempts for tackling so difficult and huge a problem as biochemical evolution, albeit of a single enzyme-type. But here and there a few indications and direction pointers have emerged, most of them leading towards a demand for greater information on the amino-acid sequences of more and more enzymes. And if the time-scale for evolution may still be wrong for a direct experimental approach (we really do not know about this) it is through the analysis of bacterial proteins at a biochemical level that we may still hope to have the best chance of obtaining some significant results.

Summary

The enzyme, generally known as "penicillinase," that hydrolyses the β-lactam bond of penicillins and cephalosporins, with production of the antibiotically inactive penicilloic and cephalosporoic acids, exists in a number of different forms, produced by a wide range of Gram-positive and Gram-negative bacterial genera (*Bacillus, Staphylococcus, Escherichia, Klebsiella,* etc.).

Despite some striking chemical and biological differences, there are probably significant resemblances between most of the varieties of penicillinase that have been studied in purified preparations. This supports the hypothesis of a common or closely related evolutionary origin.

The restricted specificity and high detoxifying activity of most penicillinases suggest that their physiological functions and evolutionary pathways are specifically related to the antibacterial action of the penicillins and cephalosporins. Moreover, the production of these antibiotics is more widespread among soil and dermatophytic microfungi than is commonly supposed, and the natural distribution of penicillin-producing micro-organisms seems to correspond fairly closely to that of penicillinase-producers.

The recent demonstration of significant penicillin production by *Penicillium* growing in soil unsupplemented by "artificial" nutrients suggests that previous scepticism regarding formation of the antibiotic "in nature" may not be justified, and that the penicillin/penicillinase relationship may be of importance in soil ecology.

Consideration of the evolutionary origin of penicillinase remains highly speculative, though it is clear that the various forms of the enzyme, as we now know them, pre-existed the therapeutic penicillin era, the only effect of which, in this context, has been to increase by natural selection the proportion of bacteria producing them.

Search for a possible penicillinase-ancestor is focused on the only other known class of protein that must, on a priori reasoning, be capable of specific combination with penicillin—that is, one or other of the enzymes involved in biosynthesis of the mucopeptide of the bacterial cell wall, a reaction known to be specifically inhibited by this antibiotic.

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