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Biochemical and genetical approaches to the mechanism of action of penicillin

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Since the discovery in 1965 that penicillin inhibits the transpeptidation reaction in peptidoglycan synthesis, a considerable effort has been put into the purification of enzymes that catalyse this reaction. This has resulted in the recognition that bacteria possess multiple forms of these penicillin-sensitive enzymes and has made it difficult to identify the precise target that penicillin inactivates to kill the organism.

Recently penicillin-sensitive enzymes have been detected and studied as penicillin-binding proteins on sodium dodecyl sulphate polyacrylamide gels. The availability of this convenient method for identifying penicillin-sensitive enzymes has allowed biochemical and genetical approaches to be used to dissect their roles in the lethal effects of penicillin and other β-lactam antibiotics. Three penicillin-binding proteins (1B, 2 and 3) have been identified as killing targets for penicillin in *Escherichia coli*, whereas four other binding proteins are not implicated in the mechanism of action of the antibiotic. The complex biological effects that β-lactam antibiotics produce on the growth of *E. coli* can be explained by their interaction with the three killing targets. Progress in the correlation of penicillin-binding proteins with penicillin-sensitive enzymes and in the development of strains of *E. coli* that overproduce penicillin-binding proteins is discussed.

Introduction

Studies on the mechanism of action of penicillin began when supplies of the antibiotic became available in the 1940s; the studies have continued actively until the present day. The early studies culminated in the discovery in 1965 that penicillin inhibited the final cross-linking reaction in peptidoglycan synthesis (Wise & Park 1965; Tipper & Strominger 1965). The enzyme that catalysed this reaction, peptidoglycan transpeptidase, was identified in cell-free extracts of *Escherichia coli* in 1966 and was shown to be inhibited by penicillin (Araki *et al.* 1966; Izaki *et al.* 1966). At this time it would not have been considered unduly optimistic to suggest that the mechanism of action of penicillin was essentially solved. Penicillin inhibited the transpeptidase enzyme and thereby prevented the synthesis of any further cross-linked peptidoglycan, resulting in the lysis of the bacterial cell and the release of the cell contents as a spheroplast. However, by the time the Royal Society held its previous symposium on penicillin in 1971 the situation no longer seemed so clear (Strominger *et al.* 1971).

By 1971 two additional penicillin-sensitive reactions had been identified (the D-alanine carboxypeptidase and peptidoglycan endopeptidase reactions; Izaki *et al.* 1966; Bogdanovsky *et al.* 1969) and bacteria had been shown to possess several distinct enzymes that catalysed these reactions (Strominger *et al.* 1971). The presence of multiple penicillin-sensitive enzymes (PSEs) in bacterial cells has raised the possibility that penicillin may inhibit bacterial growth by the inactivation of more than one enzyme and this has made it difficult to identify a 'killing target' for the antibiotic. Consideration of the biological effects of penicillin and other β-lactam antibiotics also suggests that their mechanism of killing may be complex and involve interaction with more than a single killing target. During the last few years considerable progress has been

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made in the elucidation of the role of the multiple PSEs of *E. coli* in the complex biological effects produced by β-lactams, and these will be reviewed briefly in this article. More thorough reviews of the mechanism of action of penicillin have recently been published (Blumberg & Strominger 1974; Ghuysen 1977; Spratt 1978*a*).

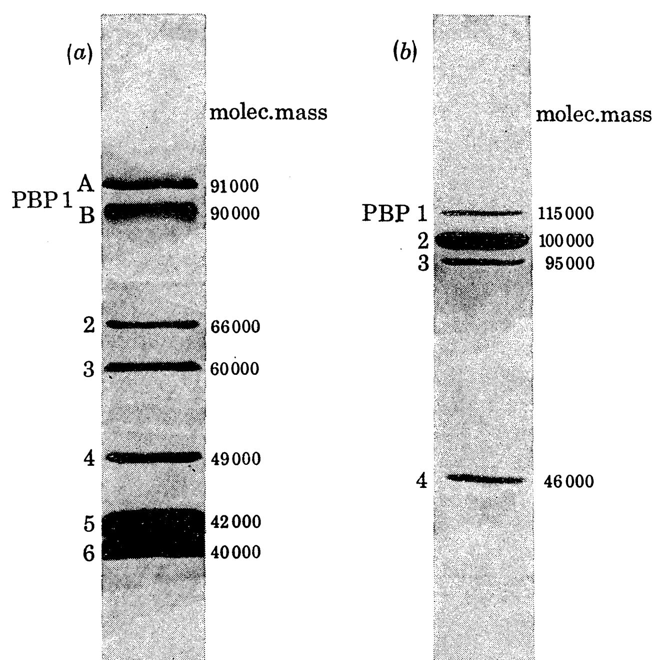

Figure 1. Radioautographic detection of the PBPs of (*a*) *Escherichia coli* and (*b*) *Staphylococcus aureus*. [^{14}C]Benzylpenicillin (30 μg/ml for *E. coli*; 3 μg/ml for *S. aureus*) was bound to cell envelopes for 10 min at 30 °C and the binding was terminated, and the release of bound penicillin prevented, by denaturation with 1 % Sarkosyl (*E. coli*) or 1 % sodium dodecyl sulphate (*S. aureus*). The PBPs were fractionated on a sodium dodecyl sulphate polyacrylamide gel and detected by scintillation radioautography (Spratt & Pardee 1975). The molecular masses of the *S. aureus* PBPs are those given by Kozarich & Strominger (1978).

The detection of penicillin-sensitive enzymes as penicillin-binding proteins

Recent progress in the identification of the *E. coli* killing targets for β-lactam antibiotics has been due largely to the development of a convenient method for the detection and study of the individual PSEs. This method derives from early work on the binding of penicillin to bacterial cells (Cooper 1956) and in particular from the mode of binding of penicillin to PSEs proposed by Tipper & Strominger (1965). They suggested that penicillin was a structural analogue of part of the peptide substrate of PSEs and that the antibiotic bound to the enzyme to form a stable penicilloyl-enzyme complex that was analogous to the transient substrate–enzyme complex formed in the normal enzyme mechanism. Strong evidence in favour of this model has accumulated over the years and it is now widely accepted (Waxman *et al.*, this symposium).

One implication of this model is that PSEs can be detected as those proteins that covalently bind radioactive penicillin (Blumberg & Strominger 1974) and can be readily visualized by scintillation radioautography after fractionation on sodium dodecyl sulphate polyacrylamide slab gels (Spratt & Pardee 1975). PSEs detected in this way are called penicillin-binding

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proteins (PBPs). All bacteria that have been examined contain between four and seven PBPs in their cytoplasmic membrane (Blumberg & Strominger 1972; Spratt & Pardee 1975; Shepherd *et al.* 1977; Kozarich & Strominger 1978; Coyette *et al.* 1978; Noguchi *et al.* 1979; Ohya *et al.* 1979). Seven PBPs with molecular masses between 40000 and 91000 have been identified in the cytoplasmic membrane of *E. coli* (Spratt & Pardee 1975; Spratt *et al.* 1977) and these are illustrated in figure 1*a*.

The usefulness of the radioautographic PBP assay depends on its ability to detect all of the PSEs of bacteria, and this depends on the assumption that all of these enzymes are inhibited by penicillin through the formation of a stable penicilloyl-enzyme complex. In practice the stability of the penicilloyl-enzyme complex is not absolute and enzymatically catalysed breakdown occurs to regenerate the active enzyme and degradation product(s) of the penicillin. The rate of breakdown varies for different enzymes and also for different β-lactams. In many cases the half life of the complex is very long (hours or days at 37 °C) but in a few cases it is fairly rapid (Ghuysen 1977; Spratt 1978*a*). (PSEs have sometimes been described as being reversibly or irreversibly inhibited by penicillin but this is not now thought to reflect any fundamental difference in their mode of inhibition but rather to reflect the stability of the penicilloyl-enzyme complex.) The most rapid breakdown of the complex that has been reported is that of a PSE with a molecular mass of 46000 from *Staphyloccocus aureus* where the half life is about 15 s at 37 °C (Kozarich & Strominger 1978). Even this enzyme can be readily detected as a PBP (PBP 4 in figure 1*b*) provided that the penicilloyl-enzyme complex is trapped by rapid denaturation with acetone (Kozarich *et al.* 1978) or with sodium dodecyl sulphate (figure 1*b*). It has been claimed that D-alanine carboxypeptidase 1B/C of *E. coli* cannot be detected as a PBP (Tamura *et al.* 1976) but the report is subject to some doubt since genetic evidence suggests that this enzyme is PBP 4 of *E. coli* (see below). Apart from this equivocal report there are no known examples of PSEs that cannot be detected as PBPs and therefore the PBP assay provides a convenient method for studying the individual properties of *all* of the PSEs of a bacterium in their normal membrane environment.

Identification of the role of the *E. coli* PBPs in the mechanism of action of penicillin

Growth of *E. coli* in the presence of β-lactam antibiotics can produce several effects on the morphology of the bacteria. With most β-lactams the lowest concentration that produces any observable response results in the inhibition of cell division and the growth of the cells into long filaments as originally described by Gardner (1940). High concentrations of almost all β-lactams result in rapid cell lysis and the release of the cell contents as a spheroplast (Duguid 1946). The third effect is produced by mecillinam, and a few other β-lactams, and is the conversion of the normal rod-shaped cells of *E. coli* into large osmotically stable spherical cells (Lund & Tybring 1972). These three morphological effects have been shown to be due to the binding of β-lactams to three of the *E. coli* PBPs. The identification of these three killing targets for β-lactams has been achieved by the use of two main methods.

First, the ability of β-lactams to elicit the three morphological effects has been correlated with their affinities for each of the *E. coli* PBPs. Examination of the morphological effects produced by a range of concentrations of many β-lactams has resulted in the identification of derivatives that are rather specific in producing a single morphological response (Spratt 1975,

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and unpublished observations). Mecillinam is a well known example. This amidinopenicillanic acid results in the growth of *E. coli* as spherical cells and at no concentration does it result in filamentation or rapid cell lysis and spheroplasting (Lund & Tybring 1972). Cefusulodin (and to a slightly lesser extent cephaloridine) results in rapid cell lysis and never produces filamentation or spherical cells. Several β-lactams cause filamentation without ever resulting in the production of spherical cells and only cause cell lysis at concentrations that are 50–100 times those that result in filamentation. Cephalexin, cefuroxime and HR756 are examples of this latter group of β-lactams.

These three types of β-lactams have been used as probes to identify the PBPs that are inactivated to result in spherical cell formation, cell lysis and filamentation (Spratt 1975), and a similar approach has recently been used to identify the equivalent PBPs in *Pseudomonas aeruginosa* (Noguchi *et al.* 1978, 1979) and *Proteus* species (Ohya *et al.* 1979).

The second approach that has proved very useful for the identification of the role of the PBPs in the lethal effects of β-lactams is that of mutant isolation. Mutants that result in the loss of activity of a PBP can be used to study the consequences of the inactivation of that PBP on the growth of the cells. Many of these mutants are likely to be non-viable and so PBP mutants have usually been screened for among temperature-sensitive mutants. So far, mutants with defects in all of the PBPs except PBP 6 have been described (Spratt 1975, 1977*b*; Spratt *et al.* 1977; Iwaya & Strominger 1977; Matsuhashi *et al.* 1977, 1978, 1979; Tamaki *et al.* 1977; Suzuki *et al.* 1978). Examination of the properties of these mutants, together with studies on the binding of β-lactams that are specific, or relatively specific, in producing a single morphological effect, has provided an understanding of the role of each of the PBPs in the killing of *E. coli* by β-lactams.

The formation of osmotically stable spherical cells has been shown to result from the binding of β-lactams to PBP 2. This was first suggested by the fact that mecillinam bound exclusively to this PBP and resulted exclusively in this morphological effect (Spratt & Pardee 1975; Spratt 1975) and has been confirmed by the demonstration that the loss of PBP 2 activity in mutants results in their growth as spherical cells that are indistinguishable from those produced by mecillinam (Spratt 1975, 1978*b*, 1979; Suzuki *et al.* 1978).

The formation of filamentous cells results from the binding of β-lactams to PBP 3. This assignment was suggested by the binding properties of β-lactams that were potent inhibitors of cell division and has been confirmed by the demonstration that the loss of the activity of PBP 3 in temperature-sensitive mutants results in their growth as long filamentous cells (Spratt 1975, 1977*b*, 1979; Suzuki *et al.* 1978).

Binding of β-lactams to PBP 1 was proposed to result in rapid cell lysis and the production of spheroplasts since there was a correlation between the ability of β-lactams to cause this effect and their affinity for PBP 1 (Spratt 1975). PBP 1 was later separated into two components, PBPs 1A and 1B, and a mutant was isolated that lacked detectable PBP 1A activity and yet grew normally (Spratt *et al.* 1977). A further eleven mutants that lack PBP 1A activity have recently been described and all of these also grow normally (Suzuki *et al.* 1978). When the affinities of PBP 1A and PBP 1B are examined, there is no correlation between binding to PBP 1A and any of the morphological effects of β-lactams. In fact this PBP binds with high affinity all β-lactams that have so far been examined. An excellent correlation is, however, found between the ability of β-lactams to cause rapid cell lysis and their binding to PBP 1B (Spratt *et al.* 1977; Spratt 1979). The proposal that the binding of β-lactams to PBP 1B results in rapid cell lysis has been supported by the isolation of a temperature-sensitive mutant that

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lacks the activity of PBP 1B *in vitro* and rapidly lyses when shifted to the restrictive temperature (Tamaki *et al.* 1977). Other mutants that lack the activity of PBP 1B *in vitro* do not show marked growth abnormalities (Suzuki *et al.* 1978). However, temperature-sensitive double mutants that lack the activities of both PBP 1A and 1B do lyse rapidly at the restrictive growth temperature (Suzuki *et al.* 1978). This has been suggested to mean that inactivation of both PBPs 1A and 1B is necessary to cause rapid cell lysis and spheroplasting. It may be that these latter mutants retain some activity of PBP 1B *in vivo* although none can be detected *in vitro*. This residual activity of PBP 1B might be sufficient for growth in the presence of an otherwise normal complement of PBPs but not in the absence of PBP 1A activity, perhaps because PBP 1A can partly compensate for the loss of PBP 1B as suggested by others (Suzuki *et al.* 1978; Tamaki *et al.* 1977). The properties of the mutant studied by Tamaki *et al.* (1977), and those of its revertants and transductants, suggest that complete inactivation of PBP 1B alone is sufficient to result in cell lysis.

PBPs 4, 5 and 6 have not been implicated in the mechanism of action of penicillin. The binding of β-lactams to these PBPs does not correlate with any of the known effects of the antibiotics. PBPs 5 and 6 have low affinities for many β-lactams (particularly cephalosporins) and in several cases inhibition of growth occurs at concentrations far below those where binding of the antibiotics to PBPs 5 and 6 can be detected (Spratt 1977*a*). These PBPs correspond to a major D-alanine carboxypeptidase (see below), and a mutant has been isolated that lacks the activity of this enzyme. The mutant grows normally under a wide range of growth conditions, which suggests that not only do these PBPs have no role in the mechanism of action of β-lactams but also that they have a non-essential role in cell growth (Matsuhashi *et al.* 1978). Several mutants that lack the activity of PBP 4 have been isolated, and these too grow normally, which suggests that this PBP is also non-essential (Iwaya & Strominger 1977; Matsuhashi *et al.* 1977). Even double mutants that lack the activity of both PBP 4 and the D-alanine carboxypeptidase that corresponds to PBPs 5 and 6 grow normally (Matsuhashi *et al.* 1978).

One problem with the mutant approach is that the inability to detect the activity of a PBP *in vitro* does not exclude the possibility that it has some activity *in vivo*. For this reason the normal growth of these mutants does not rigorously exclude some essential role for PBPs 4, 5 and 6 (and 1A), but the mutant evidence, taken together with the evidence from the binding of β-lactams to the PBPs, strongly suggests that they do not have a major role in peptidoglycan metabolism. They may, however, have a minor role in cell growth, and some subtle effects of the loss of the activity of PBPs 1A, 4 and 5 have been noted, but it is difficult to be certain that these can be ascribed to the defects in the PBPs (Iwaya & Strominger 1977; Spratt *et al.* 1977; Tamaki *et al.* 1978). Derivatives of *E. coli* that overproduce PBP 6 (see below) have been found to grow as rather rounded and irregular cells (Spratt, unpublished observations), and this suggests that alterations of PBP 6 can have some effect on the growth of the bacteria.

β-Lactam antibiotics therefore kill *E. coli* by binding to, and thereby inactivating, the PSEs that correspond to PBPs 1B, 2 and 3. The progress in the correlation of the PBPs with the known PSEs will be discussed in a later section.

The mode of action of β-lactam antibiotics seems to be similar for all Gram-negative rod-shaped organisms. The PBPs of *Salmonella typhimurium*, *Klebsiella aerogenes*, *Serratia marcescens*, several *Proteus* species and *Pseudomonas aeruginosa* have been described (Spratt 1977*c*; Shepherd *et al.* 1977; Noguchi *et al.* 1978; Ohya *et al.* 1979), and the general pattern of PBPs on polyacrylamide gels is very similar to those of *E. coli*, although slight variations occur between the

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species. These organisms all show the same range of morphological effects when exposed to β-lactams (filamentation, spherical cells, rapid cell lysis and spheroplasting) and the biochemical basis of these responses seems to be the same. Thus mecillinam converts all of these bacteria into spherical cells and in all cases binds to a single PBP with a molecular mass close to that of PBP 2 of *E. coli* (Spratt 1977*c*; Ohya *et al.* 1979; Noguchi *et al.* 1979). A recent comparison of the PBPs of *E. coli* with those of *Proteus* and *Pseudomonas* indicates that each of the PBPs of *E. coli* has a counterpart in the other bacteria that has a similar function in cell growth (Ohya *et al.* 1969; Noguchi *et al.* 1979).

Table 1. Binding to the PBPs of *E. coli* of three β-lactams that differ in their morphological effects

(Concentrations of β-lactams (micrograms per millilitre) that result in 50% saturation of PBPs†)

	mecillinam	cefsulodin	cephalexin
PBP 1A	> 500	1.0	0.9
1B	> 500	3.1	320
2	0.04	> 300	> 500
3	> 500	150	7.1
4	> 500	> 300	5.1/250‡
5	> 500	> 300	> 500
6	> 500	> 300	> 500
m.m.c.c.§/(μg/ml)	0.05	20	3
effect on growth	spherical cells	lysis and spheroplasts	filaments

† Measured by competition for binding of [^{14}C]benzylpenicillin (Spratt 1977*a*).
‡ Kinetics of binding were biphasic.
§ Mean morphological change concentration (lowest concentration resulting in morphological effect).

The killing of *E. coli* by β-lactam antibiotics

Analysis of the functions of the *E. coli* PBPs predicts that β-lactams will be effective antibiotics if they bind to either PBP 1B (possibly together with PBP 1A) or to PBP 2 or PBP 3. In practice this seems to be the case. The three antibiotics cefsulodin, cephalexin and mecillinam, which were discussed in the previous section, are examples of β-lactams that do kill by binding to only one of the killing targets. Table 1 shows the concentrations of these derivatives that are required to produce 50% saturation of each of the PBPs. Mecillinam clearly kills exclusively by binding to PBP 2, resulting in the production of spherical cells; cefsulodin only binds to PBPs 2 and 3 at high concentrations and kills by inactivation of PBP 1B, resulting in rapid cell lysis; and cephalexin kills by binding to PBP 3, causing the formation of long filamentous cells and only binds to PBPs 1B and 2 at concentrations of at least 10 times the minimal inhibitory concentration. These three β-lactams have therefore completely different mechanisms of action. Antibiotics that have related but distinct mechanisms of action sometimes act synergistically; this has been found with some combinations of β-lactams (e.g. mecillinam plus ampicillin; Tybring & Melchior 1975).

Although β-lactams can kill by the inactivation of a single killing target, many of them have rather similar affinities for two or all three of the targets, and in these cases the morphological effects that they produce can be explained by their relative affinities for the targets (Spratt 1975, 1978*a*). As far as we know, the binding of β-lactams to PBPs 4, 5 and 6 is completely irrelevant to their efficacy as antibiotics.

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The most potent β-lactams should be those that have very high affinity for PBP 1B, since the rate of killing after inactivation of this target is rapid. At present no derivatives with very high affinity for this PBP have been found, and the best of them show 50% saturation of PBP 1B at about 1 μg/ml. On the other hand, several β-lactams are known that show 50% saturation of PBP 2 or PBP 3 at concentrations of less than 0.05 μg/ml (e.g. mecillinam and thienamycin for PBP 2, and HR756 for PBP 3), and these are currently the most active derivatives against *E. coli*.

Although it is often stated that β-lactams kill bacteria by inhibition of cross-linking through the inactivation of peptidoglycan transpeptidase, this is clearly only partly true at least for *E. coli*. The major transpeptidase of *E. coli* is almost certainly PBP 1B (see below) and therefore the many β-lactams that kill by inactivation of PBP 3 or PBP 2 (e.g. cephalexin, cefuroxime, mecillinam) do so without any inhibition of transpeptidase. Inactivation of transpeptidase is lethal, resulting in rapid cell lysis, but is not essential for the killing of *E. coli* by β-lactam antibiotics.

Correlation of penicillin-sensitive enzymes with penicillin-binding proteins

Studies by Ward and his colleagues on peptidoglycan synthesis in *Bacillus licheniformis* have shown that peptidoglycan precursors are polymerized to form long linear glycan chains (nascent peptidoglycan), which are incorporated into the pre-existing peptidoglycan by the penicillin-sensitive transpeptidation reaction (Ward & Perkins 1974). This mechanism of synthesis has also been shown to occur in *E. coli* (Mirelman *et al.* 1976). Inactivation of the transpeptidase that performs this function *in vivo* is therefore expected to block completely the formation of peptidoglycan, leading to the accumulation of nascent peptidoglycan and resulting, either directly or through the action of autolytic enzymes, in rapid cell lysis and the release of the bacterial cell contents as a spheroplast at the site of blocked cell wall synthesis. This is precisely the response of *E. coli* to growth in the presence of cefsulodin or cephaloridine, or indeed to high concentrations of virtually all β-lactam antibiotics. As I discussed above, this response is due to the inactivation of PBP 1B and it would therefore appear that this PBP is the major physiologically important transpeptidase of *E. coli*.

The transpeptidation reaction can be measured in crude cell extracts by following the incorporation of peptidoglycan precursors into cross-linked peptidoglycan (Izaki *et al.* 1966). Studies of the concentrations of β-lactams that inhibit crude transpeptidase activity by 50% correlate fairly well with the concentrations required to give 50% saturation of PBP 1B (Oka & Fujita 1978). Mutants that lack the activity of PBP 1B also provide additional evidence that this protein is the major transpeptidase of *E. coli*. These mutants show very low levels of peptidoglycan synthesis *in vitro* which makes measurement of the level of transpeptidation difficult, but in the one case where it has been measured it was about 10% of the level in normal cells (Matsuhashi *et al.* 1978).

The low level of peptidoglycan synthesis *in vitro* in PBP 1B mutants suggests that both transpeptidation and the synthesis of nascent peptidoglycan are being inhibited. This is unexpected, since the synthesis of nascent peptidoglycan by extracts of wild-type cells is not greatly inhibited even by high concentrations of β-lactams (Izaki *et al.* 1966). One possible explanation of this anomaly is that the transpeptidase (PBP 1B) forms part of a complex in the inner membrane which catalyses the several enzymatic reactions involved in the incorporation of peptidoglycan

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precursors into cross-linked peptidoglycan. The transpeptidase activity found by Izaki *et al.* (1966) depends on the activity of the whole complex, and it may be that the subtle alteration of PBP 1B produced by the binding of a β-lactam antibiotic has little effect on the activity of the other enzymes in the complex, so that the synthesis of nascent peptidoglycan can continue undisturbed. On the other hand, a much more drastic alteration of the conformation of PBP 1B may occur in the mutants, resulting in the loss of activity of the whole peptidoglycan-synthesizing complex. This, of course, is only speculation and more studies of these interesting mutants are needed to understand the role of PBP 1B in peptidoglycan synthesis.

It is not clear whether the transpeptidase activity in extracts of *E. coli* is due exclusively to PBP 1B or whether other PBPs make some contribution to this activity. The two major D-alanine carboxypeptidases of *E. coli* (see below) have been shown to catalyse weak model transpeptidase activity (Nguyen-Distèche *et al.* 1974; Tamura *et al.* 1976). For example, they catalyse the replacement of the terminal D-alanine of UDP-*N*-acetylmuramyl pentapeptide by glycine. However, the demonstration that PSEs or PBPs catalyse a model transpeptidase reaction once purified is no proof that they catalyse transpeptidation *in vivo*, or that they will contribute to the transpeptidase assay measured in the natural coupled system used by Izaki *et al.* (1966). The latter assay may well only detect the activity of those enzymes that act as transpeptidases *in vivo*, since it presumably depends on the intimate association of the transpeptidase with the other membrane-bound enzyme steps that are involved in the incorporation of peptidoglycan precursor into cross-linked peptidoglycan.

The D-alanine carboxypeptidase 1 reaction was first detected in crude extracts of *E. coli* by Izaki *et al.* (1966). This activity is largely due to two major carboxypeptidases which have been purified from *E. coli* membranes (Tamura *et al.* 1976; Nguyen-Distèche *et al.* 1974). One of these enzymes, D-alanine carboxypeptidase 1A, is moderately penicillin-sensitive and forms a protein doublet on sodium dodecyl sulphate polyacrylamide gels which corresponds to PBPs 5 and 6 (Spratt & Strominger 1976). Only one mutant that lacks the activity of this enzyme has been described and has been shown to have an altered PBP 5 (Matsuhashi *et al.* 1979). It is not yet clear whether alteration of PBP 6 will also result in the loss of D-alanine carboxypeptidase 1A activity and the precise relation between the enzyme and PBPs 5 and 6 remains unclear. PBPs 5 and 6 are certainly distinct proteins, rather than being modified forms of the same gene product, since they have been independently cloned onto phage λ and pSF2124 plasmid respectively (Spratt, unpublished results). The simplest explanation is that the enzyme is a complex consisting of subunits of PBPs 5 and 6.

The second enzyme, D-alanine carboxypeptidase 1B/C, is the major carboxypeptidase of *E. coli* and is highly penicillin-sensitive. It has been reported that the enzyme fails to bind [^{14}C]-benzylpenicillin covalently. This result needs to be reinvestigated since a considerable amount of evidence has accumulated to suggest that D-alanine carboxypeptidase 1B/C is PBP 4. For example, several independent mutants have been reported that lack carboxypeptidase 1B/C and have simultaneously lost PBP 4 activity (Iwaya & Strominger 1977; Matsuhashi *et al.* 1977). Furthermore, PBP 4 has been purified from *Salmonella typhimurium* and has been shown to be the major D-alanine carboxypeptidase 1 of the organism and to have properties very similar to those of D-alanine carboxypeptidase 1B/C of *E. coli* (Shepherd *et al.* 1977).

D-Alanine carboxypeptidase 1 activity has often been suggested to function in regulating the level of cross-linking in the peptidoglycan by converting some of the pentapeptide side chains of the nascent peptidoglycan into tetrapeptides and thereby preventing them from being

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involved as peptide donors in cross-linking (Izaki *et al.* 1966; Blumberg & Strominger 1974). Extracts of the double mutant that lacks the activities of D-alanine carboxypeptidase 1A and 1B/C show extremely low levels of carboxypeptidase activity and it might be expected that the peptidoglycan of these mutants would show an abnormally high level of cross-linking. In practice they do not (Matsuhashi *et al.* 1978). This could be taken to mean that the two major D-alanine carboxypeptidases of *E. coli* are not involved in the regulation of the level of peptidoglycan cross-linking and that this is either the function of a minor carboxypeptidase (perhaps one of the minor PBPs) or is determined by some completely different mechanism. Unfortunately, as discussed above, it is not certain that these mutants have no D-alanine carboxypeptidase activity *in vivo*.

The third penicillin-sensitive activity that has been detected in cell extracts of *E. coli* is that of peptidoglycan endopeptidase (Bogdanovsky *et al.* 1969). This activity is due to D-alanine carboxypeptidase 1B/C, which catalyses a strong endopeptidase reaction in addition to its carboxypeptidase activity (Tamura *et al.* 1976; Nguyen-Distèche *et al.* 1974; Matsuhashi *et al.* 1977). Loss of the activity of this enzyme by mutation results in the loss of all of the penicillin-sensitive endopeptidase activity of cell extracts (Matsuhashi *et al.* 1977).

In summary, *E. coli* is known to possess a transpeptidase which is thought to be PBP 1B, a carboxypeptidase–endopeptidase which is PBP 4 and a second carboxypeptidase which is PBPs 5 and 6. Nothing is known of the enzymatic functions of PBP 1A or of the two killing targets, PBPs 2 and 3. Presumably these PBPs catalyse one or more of the known penicillin-sensitive reactions but make too minor a contribution to the total activity of cell extracts to be readily detected.

Genetic approaches to the overproduction of penicillin-binding proteins

At present little progress in the purification of PBPs 1A, 1B, 2 or 3 has been reported. Clearly the purification and study of the killing targets (PBPs 1B, 2 and 3) is an important goal for future research. Purification of these minor components of the cytoplasmic membrane would be simplified if their relative abundance in the membrane could be increased. This can be achieved by raising the number of copies of PBP genes in the cell by inserting them into multicopy plasmids by *in vitro* recombinant DNA techniques. As an example, figure 2*b* shows the PBPs of *E. coli* carrying the plasmid pLG310. This plasmid was constructed by the insertion of a 3.9×10^6 molecular mass EcoR1 restriction enzyme fragment containing the PBP 6 gene into the multicopy plasmid cloning vector pSF2124 (B. G. Spratt & R. Diaz, unpublished experiments). Overproduction of PBP 6 results from the presence of between 30 and 50 copies of the PBP 6 gene in the cells. Nishimura *et al.* (1977) have identified a co1E plasmid containing the PBP 3 gene (pLC26-6) in the Carbon–Clarke collection of cloned fragments of the *E. coli* genome (Clarke & Carbon 1976). Figure 2*d* shows the overproduction of PBP 3 in a strain carrying this plasmid.

The level of overproduction of PBPs produced by this method is relatively modest and an alternative method is to introduce the PBP genes onto bacteriophage λ. Overproduction in this case can occur as multiple copies of the gene accumulate during the lytic development of the phage. We have recently isolated λ phage carrying the PBP 2 and PBP 5 genes. Figure 2*f* shows the increased level of PBP 2 in the cytoplasmic membrane after induction of *E. coli* lysogenic for λ carrying the PBP 2 gene. The level of other PBPs decreases and this is probably

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due to the reduction in the expression of the chromosomal PBP genes during λ development. In this preliminary experiment the overproduction of PBP 2 is not great since the packaging of the phage DNA into phage heads occurs and prevents further transcription of the PBP 2 genes. The introduction of mutations that prevent the switching on of λ late functions should allow continued expression from the multiple copies of the PBP 2 genes since packaging into phage heads is prevented. Moir & Brammar (1976) have shown the massive levels of overproduction of the products of genes carried on λ phage that can result from this approach

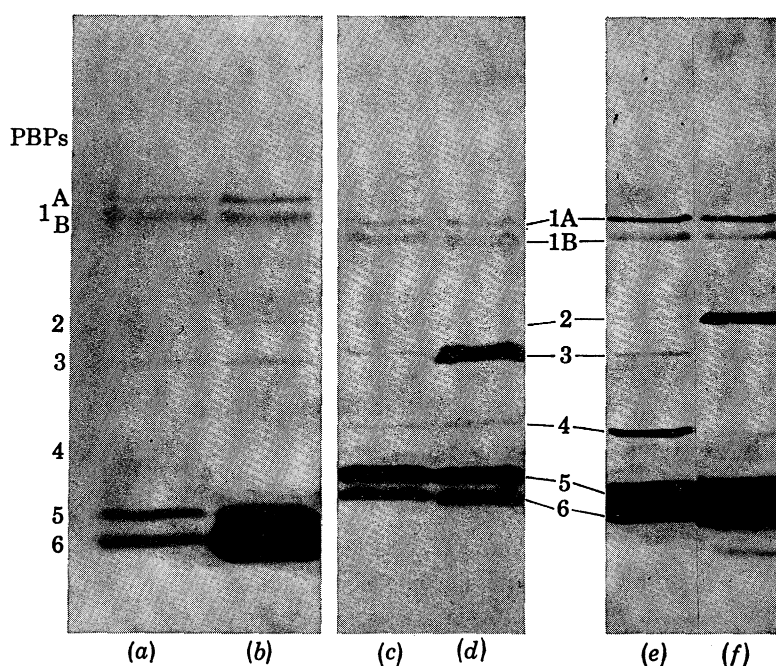

FIGURE 2. Overproduction of *E. coli* PBPs: the PBPs of *E. coli* (*a*) and a derivative containing the pLG310 plasmid (*b*) (the *E. coli* used in (*a*) and (*b*) was a mutant that lacks PBP 4 activity); PBPs of *E. coli* (*c*) and a derivative containing the pLC26-6 plasmid (*d*); PBPs of *E. coli* lysogenic for phage λ carrying the PBP 2 gene before (*e*) and after phage induction (*f*).

CONCLUSIONS

Fifty years after Fleming's discovery of penicillin we can claim to know with reasonable certainty the killing targets for the antibiotic in *E. coli* and to understand the biological effects produced by β-lactams in terms of their interaction with these targets. We still know little about the enzymology of the killing targets, since none of them have been purified, although of course much is known of the properties of penicillin-sensitive enzymes from the extensive studies of those enzymes that have been purified. However, there is no clear evidence that any of these latter enzymes are killing targets for β-lactams.

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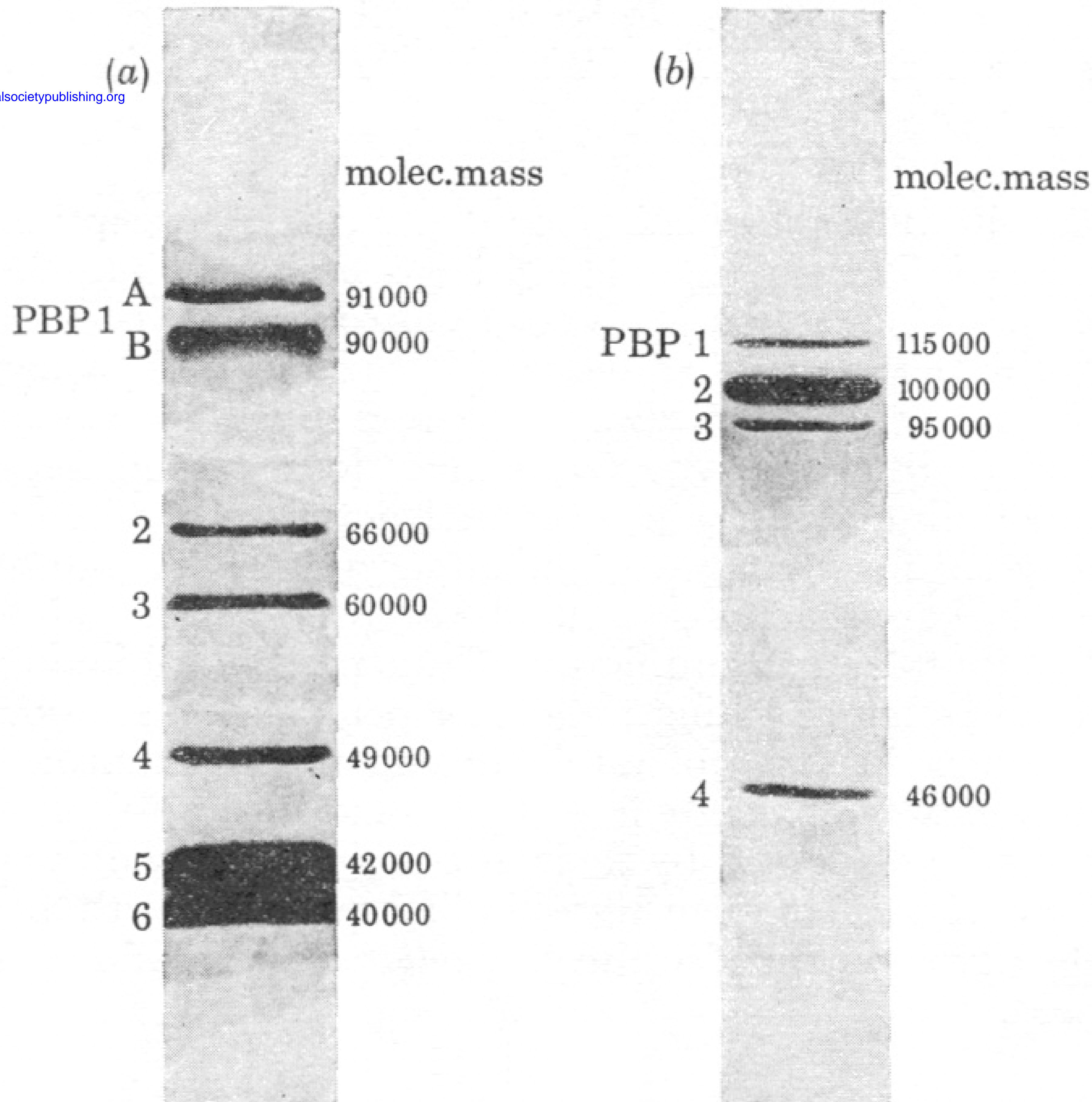

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GURE 1. Radioautographic detection of the PBPs of (*a*) *Escherichia coli* and (*b*) *Staphylococcus aureus*. [^{14}C]Benzylpenicillin (30 µg/ml for *E. coli*; 3 µg/ml for *S. aureus*) was bound to cell envelopes for 10 min at 30 °C and the binding was terminated, and the release of bound penicillin prevented, by denaturation with 1 % Sarkosyl (*E. coli*) or 1 % sodium dodecyl sulphate (*S. aureus*). The PBPs were fractionated on a sodium dodecyl sulphate polyacrylamide gel and detected by scintillation radioautography (Spratt & Pardee 1975). The molecular masses of the *S. aureus* PBPs are those given by Kozarich & Strominger (1978).

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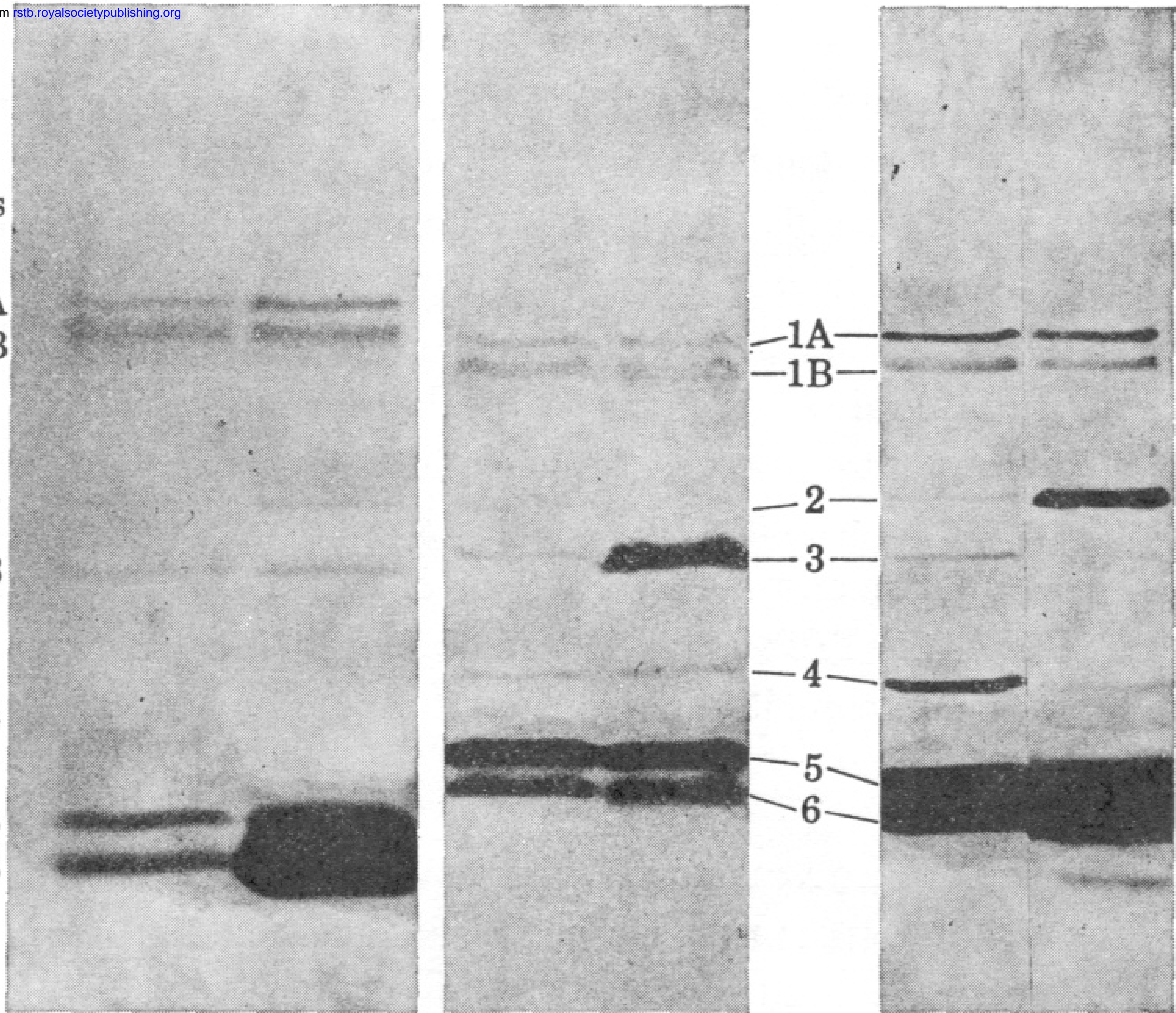

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GURE 2. Overproduction of *E. coli* PBPs: the PBPs of *E. coli* (*a*) and a derivative containing the pLG310 plasmid (*b*) (the *E. coli* used in (*a*) and (*b*) was a mutant that lacks PBP 4 activity); PBPs of *E. coli* (*c*) and a derivative containing the pLC26-6 plasmid (*d*); PBPs of *E. coli* lysogenic for phage λ carrying the PBP 2 gene before (*e*) and after phage induction (*f*).