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Reaction of ampicillin with serum albumin to produce penicilloyl-protein conjugates and a piperazinedione

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Formation of penicilloyl-protein conjugates in the body by reaction of penicillins with nucleophilic groups of proteins is considered to be involved in penicillin allergy. In this study the kinetics and mechanism of reaction of ampicillin with human and bovine serum albumin in aqueous solution at 37 °C and pH 6·6–10·4 have been investigated and compared with the reaction of benzylpenicillin previously reported. In addition to forming penicilloyl-protein conjugates, ampicillin was found to react with the proteins to yield a free piperazine-2,5-dione derivative. This product is suggested to arise from intramolecular aminolysis of an *N*-(penicilloyl)imidazole intermediate at pH 6–8 and of a penicilloyl ester intermediate at higher pH values. The piperazinedione formation was found to compete effectively with formation of penicilloyl-protein conjugates at physiological pH and, along with studies of ampicillin with protein model compounds, the reaction allowed suggestions to be made about the penicillin-reacting sites in the proteins.

It is generally agreed that the principal antigenic determinant in penicillin allergy is the penicilloyl group. This can be introduced to the body as pre-formed penicilloyl-protein conjugate impurities surviving the manufacturing process, penicillin polymers and other penicilloyl derivatives formed upon in vitro degradation of the drugs, or it can be formed in vivo by an irreversible reaction of penicillins with nucleophilic groups of tissue proteins, especially ε-amino groups of lysine residues (for reviews, see Schneider 1970; Dewdney 1977; Bundgaard 1980; Ahlstedt et al 1980). Although considerable controversies still exist concerning the relative importance of these routes to the formation of immunogenic and antigenic penicilloyl conjugates, penicilloyl-protein conjugates either introduced as impurities or formed in vivo are thought to play a major role in penicillin allergy.

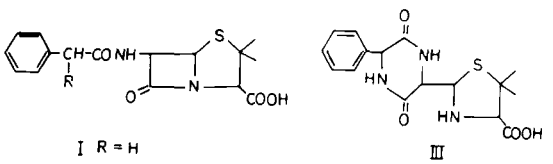
Penicillins have been shown capable of reacting irreversibly with various proteins such as serum proteins (Batchelor et al 1965; Horiuchi & Shibata 1965; Schneider & de Weck 1966; Bundgaard 1977) and proteins of various cell membranes (de Weck 1964; Josephson & Kaplan 1967; Naor et al 1971; Avtalion & Milgrom 1976) in neutral and alkaline aqueous media, but an understanding of which proteins or other macro-molecules react with penicillin in vivo is still lacking as is the mechanism of reaction. Serum albumin may possibly be the carrier or one of the carriers to which penicilloyl groups become attached upon administration of penicillin. Thus, it has been reported (Wagner et al 1973; Girard & Cuevas 1975) that upon incubation of benzylpenicillin with human or guinea-pig serum at physiological conditions of pH and temperature, 70–80% of the covalently bound penicillin is found in albumin. With a specific radioimmunoassay, evidence has recently been shown of in vivo formation of penicilloylated serum albumin following parenteral administration of benzylpenicillin in animals as well as in man (Lapresle & Wal 1979; Wal 1980).

A kinetic study (Bundgaard 1977) of the reaction of human (HSA) and bovine serum albumin (BSA) with benzylpenicillin and various other penicillins at 37 °C showed that the penicilloylation of the proteins occurred almost exclusively with the ε-amino groups of lysine residues and that the rate of reaction at alkaline pH was in agreement with a direct penicilloylation of these groups, reacting independently of each other. At pH 6–8, however, the rate was found to be 10 times higher than could be explained by a reaction with ε-amino groups. The kinetic analysis indicated that the rate-determining step at physiological pH was a reaction with a second nucleophile having a pK_a value of 7·2. This was interpreted as a reaction with the histidine imidazole groups with formation of reactive *N*-(penicilloyl)imidazole derivatives which could then transfer the penicilloyl groups to neighbouring acceptor amino groups.

We now present results that support this interpretation of the mechanism of penicilloylation of serum albumin at physiological pH and show that the reac-

* Correspondence.

tion of ampicillin (II) with protein is different from that of other penicillins such as benzylpenicillin (I).



I R = H
II R = NH_2

III

MATERIALS AND METHODS

Materials and apparatus

Ampicillin sodium was a commercial product with a purity better than 97%. The piperazine-2,5-dione derivative (III) of ampicillin was available from a previous study (Bundgaard & Larsen 1979). Bovine and human serum albumin were purchased from Sigma Chemical Co, St Louis, U.S.A. Crystallized, 'fraction V powder' and 'essentially fatty acid-free' preparations were used. All other chemicals used were of reagent grade. Ultraviolet spectral measurements were performed with a Perkin-Elmer 124 spectrophotometer, using 1 cm cuvettes. The pH-stat employed was a Radiometer SBR 2/SBU 1 Titrigraph in conjunction with a Radiometer TTT 11 automatic titrator and a Radiometer model PHM 26 pH meter equipped with a scale expander.

Analytical procedures

The concentrations of penicilloyl derivatives (amides or esters) (V and VI, Scheme 1), the piperazine-2,5-dione derivative and penicilloic acid in the reaction solutions were determined by means of the spectrophotometric penamaldate assay as previously described (Bundgaard & Larsen 1979). This assay allows the simultaneous determination of all these products and is also applicable for the direct determination of covalently bound penicilloyl groups in proteins (Bundgaard 1977). The piperazine-2,5-dione derivative was further quantitated by the spectrophotometric assay recently described (Bundgaard & Hansen 1981). This assay involves conversion of the piperazine-2,5-dione into a highly absorbing (λ_{max} 322 nm) product by treatment with 1 M sodium hydroxide for 3–4 min at room temperature; no interference was made by ampicillin or its penicilloic acid and penicilloyl amide derivatives.

Kinetic measurements

Reactions of ampicillin with the proteins HSA and BSA were carried out in aqueous solutions at 37 ± 0·1 °C and were studied at various penicillin and protein concentrations in the pH-interval 6·6–10·4 using a pH-stat to keep constant pH during the reactions. Protein solutions were prepared gravimetrically and in calculating the molar concentrations of HSA and BSA a molecular weight of 66 000 was used (Spahr & Edsall 1964). The kinetics of the reactions was generally determined on the basis of measurement of initial rates of piperizone-2,5-dione and penicilloyl-protein conjugate formation. The reactions were initiated by adding an accurately weighed quantity of ampicillin sodium to a known volume of the aqueous protein solution pre-equilibrated at 37 °C. At appropriate intervals, samples of the reaction solutions were removed and, after suitable dilution with a 0·1 M phosphate buffer of pH 7·0, analysed by means of the penamaldate assay and the spectrophotometric assay for piperazine-2,5-dione. In some cases the kinetics of reaction was also determined on the basis of the entire course of reaction. Calculation of rate constants from the experimental data was done as previously described (Bundgaard 1977).

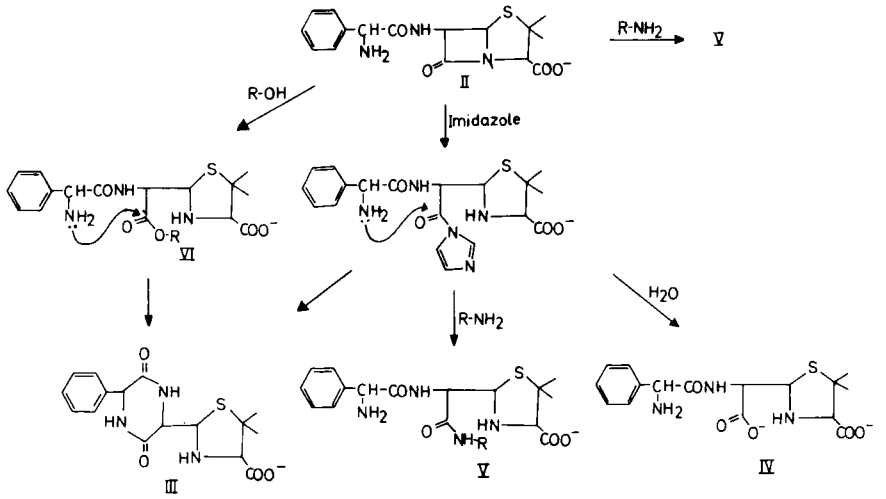
Reactions with model compounds

Reactions of ampicillin with imidazole, phenol and glycine, alone or in admixture, were carried out at 37 °C, the initial ampicillin concentration being 4×10^{-3} M. Completed reaction solutions were analysed for penicilloyl amide, penicilloic acid and piperazine-2,5-dione derivative as described above.

RESULTS AND DISCUSSION

Reaction of ampicillin with amine and oxygen nucleophiles

Aminolysis of penicillins by imidazole is an efficient process at neutral pH (Bundgaard 1971, 1972a, b, 1976b). Although the final products of reaction of penicillins with imidazole are penicillenic acid and penicilloic acid, the reaction proceeds quantitatively through *N*-(penicilloyl)imidazole (Bundgaard 1972a, 1976a). This highly reactive intermediate can readily transfer its penicilloyl group to amino and hydroxyl groups with the formation of more stable penicilloyl compounds (Bundgaard 1972c, 1976a; Yamana et al 1975a). In the reaction of ampicillin with imidazole, however, a stable piperazine-2,5-dione derivative (III) was found to be formed in addition to the product of hydrolysis, penicilloic acid (IV). As seen from Table 1 the amount formed was about 60% over the pH-range 6·1–7·7. Isolation and identification of the product were accomplished as described in a recent study on phosphate-catalysed transformation of ampicillin to the piperazine-2,5-dione derivative (Bundgaard & Hansen 1981). In accord with the findings reported in that study the



Scheme 1

imidazole-catalysed formation of III may most likely occur by a rapid intramolecular aminolysis of the *N*-(penicilloyl)imidazole intermediate (Scheme 1). Penicillins lacking a side-chain amino group are structurally unable to rearrange into a piperazine-2,5-dione derivative. This finding of imidazole-catalysed transformation of ampicillin to a piperazinedione explains the anomalous behaviour observed with ampicillin in the imidazole-assay for penicillins (Bundgaard & Ilver 1972; Bundgaard 1974).

Reaction of ampicillin with phenol also results in the formation of appreciable amounts of III (Table 1). It was found by Bundgaard & Larsen (1979) that the piperazine-dione (III) is a major product of reaction of ampicillin with various alcohols including carbohydrates, the product being formed by an intramolecular aminolysis of a penicilloyl ester intermediate (VI). In contrast to the reactions with imidazole and hydroxy compounds no piperazinedione is produced in reactions of ampicillin with primary and secondary amines, the major reaction product being stable penicilloyl amides (V) (Table 1).

Table 1. Products of reaction of ampicillin with imidazole, glycine or phenol in aqueous solution at 37 °C.

Reaction conditions*	Products (mol %)		
	Piperazine-2,5-dione (III)	Penicilloic acid	Penicilloyl amide
0·25 M Imidazole pH 6·14	59	41	0
0·25 M Imidazole pH 6·75	60	39	0
0·25 M Imidazole pH 7·09	60	38	0
0·25 M Imidazole pH 7·40	55	45	0
0·25 M Imidazole pH 7·70	54	47	0
0·5 M Glycine pH 9·99	0	3	97
0·5 M Phenol pH 9·97	60	40	0

* The initial ampicillin sodium concentration was 4×10^{-3} M.

To determine whether the imidazole-catalysed rearrangement of ampicillin to the piperazinedione may compete with the imidazole-catalysed penicilloylation of an acceptor amine, ampicillin was allowed to react with imidazole at pH 7·40 in the presence of various concentrations of glycine. As shown in Fig. 1 the percentage yield of the formed penicilloyl amide is markedly lower than that observed for benzylpenicillin (Bundgaard 1976a) under similar reaction conditions. The reactivity of ampicillin and benzylpenicillin with imidazole is almost identical (Bundgaard 1976b) and the results obtained therefore show that intramolecular aminolysis of the

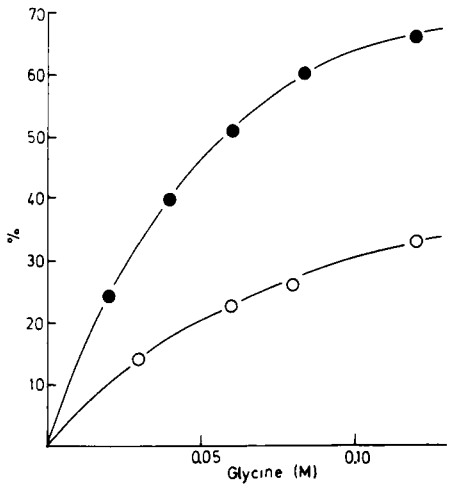


FIG. 1. Effect of glycine concentration on the percentage yield of penicilloyl amides formed by reaction of ampicillin (○) and benzylpenicillin (●) (4×10^{-3} M) with imidazole (0·025 M) at pH 7·40 and 37 °C. The data for benzylpenicillin are from Bundgaard (1976a).

N-(α-aminobenzylpenicilloyl)imidazole intermediate to yield III is capable of competing with but not excluding intermolecular aminolysis of the intermediate to yield *N*-(penicilloyl)glycine (Scheme 1).

Reaction of ampicillin with serum albumin

Besides producing penicilloyl conjugates and the hydrolysis product, α-aminobenzylpenicilloic acid, reaction of ampicillin with HSA or BSA in aqueous solution at 37 °C resulted in the formation of the piperazinedione III. This product was identified and quantitated as described above and a sample was isolated by acidifying a completed reaction solution (pH 7·4) to pH 2 followed by extraction with ethyl acetate and evaporation of solvent.

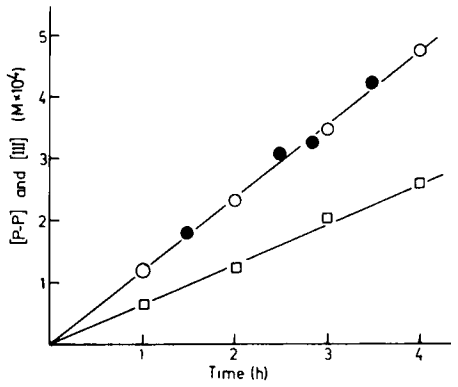


FIG. 2. Plots showing initial rates of formation of penicilloyl-protein conjugates (□) and piperazinedione (○) in reaction of ampicillin (3×10^{-2} M) with BSA (0·6 mM) in aqueous solution at pH 7·30 and 37 °C as determined by the penamaldate assay. The symbols ● represent piperazinedione determined by the method with sodium hydroxide.

The initial rates of the simultaneous formation of penicilloyl-protein conjugates and piperazinedione were followed at 37 °C as a function of ampicillin and protein concentration and pH. As shown in Fig. 2, plots of formed penicilloyl conjugate or piperazinedione versus time yielded straight lines, from the slopes of which the initial rates were calculated as previously reported (Bundgaard 1977). At constant pH and temperature, both reactions were first-order with respect to both ampicillin and protein concentration over the ranges studied (0·01–0·04 M and 0·2–0·8 mM, respectively). Thus the reactions can be described by the rate expressions:

$$\frac{d[P\text{-}P]}{dt} = k_1[Amp][Protein] \quad (1)$$

$$\frac{d[III]}{dt} = k_2[Amp][Protein] \quad (2)$$

where [Amp] and [Protein] represent the molar concentrations of ampicillin and HSA or BSA, respectively, P-P represents penicilloyl-protein conjugate and k_1 and k_2 are pH-dependent second-order rate constants for penicilloylation and piperazinedione formation, respectively. Values of these rate constants, derived by dividing the initial rates with penicillin and protein concentration, were obtained at different pH values and are listed in Table 2. The various preparations of HSA and BSA showed the same reactivity (within ±10%).

Table 2. Second-order rate constants for formation of penicilloyl-protein conjugates (k_1) and piperazinedione III (k_2) by reaction of ampicillin with BSA in aqueous solution at 37 °C.

pH	k_1 (M^{-1} min^{-1})	k_2 (M^{-1} min^{-1})
6·59	0·014	0·042
6·75	0·025	0·060
7·30	0·060	0·11
8·14	0·20	0·13
8·91	0·60	0·20
9·55	2·0	0·40
10·01	5·3	0·85
10·41	10·3	1·19

In some cases (runs at pH 7·30 and 9·55), the reactions were also followed to completion. Both reactions displayed good pseudo-first-order kinetics and by transforming the rate constants thus obtained to the second-order rate constants k_1 and k_2 in a manner previously described (Bundgaard 1977), values were derived which corresponded well (±15%) with those obtained from determination of initial rates. In these runs about 4 (at pH 7·30) and 7 (at pH 9·55) penicilloyl groups, on the average, conjugated to one protein molecule and the pseudo-first-order kinetics observed thus indicate that these protein reaction sites (and the sites catalysing the piperazinedione formation) apparently react with ampicillin at the same rate and independently of each other as has previously been observed for benzylpenicillin and cloxacillin (Bundgaard 1977).

Plots of pH versus the logarithm of the rate constants k_1 and k_2 for the reaction of ampicillin with BSA are presented in Fig. 3 together with the pH-log k_1 profile for benzylpenicillin previously derived (Bundgaard 1977). The variation in the rates of penicilloylation and piperazinedione formation with pH can be accounted for by assuming reactions involving the free base forms of nucleophilic groups

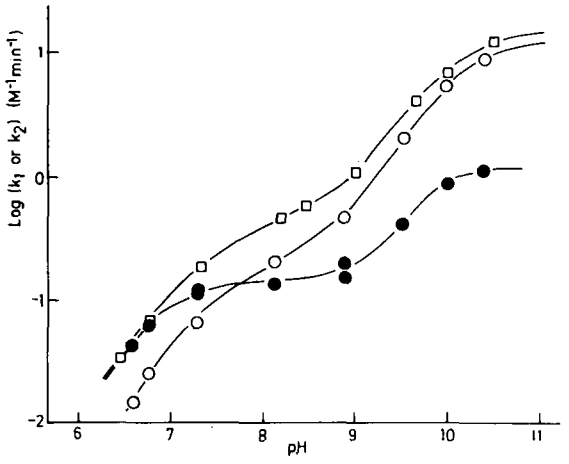


FIG. 3. Plots of log k_1 and log k_2 versus pH for formation of penicilloyl-protein conjugates (k_1) (○) and piperazinedione (k_2) (●) in reaction of ampicillin with serum albumin in aqueous solution at 37 °C. The pH-rate profile for penicilloylation of albumin by benzylpenicillin (□) is included for comparison and is from Bundgaard (1977).

with the ionization constants K_a' and K_a'' (or K_a^* and K_a^{**}) according to equations (3) and (4):

$$k_1 = \frac{k_1'K_a'}{a_H + K_a'} + \frac{k_1''K_a''}{a_H + K_a''} \quad (3)$$

$$k_2 = \frac{k_2'K_a^*}{a_H + K_a^*} + \frac{k_2''K_a^{**}}{a_H + K_a^{**}} \quad (4)$$

in which a_H is the hydrogen ion activity, and k_1' and k_1'' are pH-independent second-order rate constants for penicilloylation of the nucleophilic reaction sites on the protein molecule and k_2' and k_2'' are pH-independent second-order rate constants for formation of the piperazinedione derivative by catalysis by nucleophilic sites with the ionization constants K_a^* and K_a^{**}. In Fig. 3 the points are experimental and the curves constructed from the most satisfactory solution of equations (3) and (4). The following values of rate and ionization constants were derived and used in constructing the curves:

$k_1' = 0{\cdot}12$ M^{-1} min^{-1}; $pK_a' = 7{\cdot}2$
$k_1'' = 14.0$ M^{-1} min^{-1}; $pK_a'' = 10{\cdot}2$
$k_2' = 0{\cdot}16$ M^{-1} min^{-1}; $pK_a^* = 7{\cdot}0$
$k_2'' = 1{\cdot}25$ M^{-1} min^{-1}; $pK_a^{**} = 10{\cdot}0$

For comparison the corresponding constants for penicilloylation of serum albumin by benzylpenicillin are (Bundgaard 1977):

$k_1' = 0{\cdot}27$ M^{-1} min^{-1}; $pK_a' = 7{\cdot}2$
$k_1'' = 16{\cdot}8$ M^{-1} min^{-1}; $pK_a'' = 10{\cdot}2$

In the previous study with benzylpenicillin (Bundgaard 1977) the penicillin-reactive groups of pK_a 10·2 were suggested to be ε-amino groups of lysine residues while the penicillin-reacting sites having the apparent pK_a of 7·2 were attributed to imidazole groups of histidine residues. These conclusions were made on basis of the pK_a values as well as by comparison of the rate data with those for simple model compounds of functional groups of proteins.

The results of the present study with ampicillin provide supporting evidence for and extend this interpretation. The rate data for penicilloylation of BSA by ampicillin and benzylpenicillin are comparable except that the k_1' value is reduced for ampicillin. This reduction is due to a competing reaction with the protein leading to formation of the piperazinedione derivative III. The reaction sites being responsible for this reaction at pH 6–8 possess an apparent pK_a value of 7·0 which, within experimental error, is similar to the pK_a value of the sites reacting with formation of penicilloyl-protein conjugate. From the results obtained on reaction of ampicillin with the protein model compounds the reaction behaviour and the pK_a value (7·0) of imidazole provide strong support for the view that the penicillin-reacting sites of pK_a 7–7·2, both as regards penicilloylation and piperazinedione formation, are imidazole groups of histidine residues. Thus, the reaction of the penicillins with protein at physiological pH may be depicted as shown in Scheme 1 with *N*-(penicilloyl)-imidazole groups being a reactive intermediate, formed in the rate-determining step. Additional evidence is provided by the fact that the sum of the rate constants k_1' and k_2' is equal to k_1' for benzylpenicillin.

The reaction sites of the protein molecule accounting for the protein-catalysed formation of the piperazinedione product at pH > 9 and possessing an apparent pK_a of 10·0 may most likely be phenol groups of tyrosine residues although hydroxyl groups of threonine or serine residues cannot be excluded. As described above α-aminobenzylpenicilloyl esters formed by reaction of ampicillin with phenol and alcohols readily undergo intramolecular aminolysis to yield piperazinedione. Lysine ε-amino groups are not implicated as the reaction sites since the model experiments showed *N*-(α-aminobenzyl-penicilloyl)glycine to be incapable of rearranging to the piperazinedione. The formation of piperazinedione from a reaction site of pK_a 10·0 may imply, on the other hand, that this site is also involved in the formation of penicilloyl-protein conjugate since piperazinedione formation relies on a penicilloyl intermediate (imidazolide or ester). Thus, the reaction sites with pK_a of 10–10·2 being predominantly

responsible for penicilloylation of protein at pH > 9 are suggested to be both lysine ε-amino groups and, to a minor extent (about 10%), hydroxyl groups of tyrosine, serine or threonine residues. It has previously been shown that such hydroxyl groups in model compounds are nearly as reactive with benzylpenicillin as ε-amino groups (Bundgaard 1976b; Bundgaard & Larsen 1978) and that penicilloyl esters may transfer their penicilloyl groups to closely located amino groups to yield stable penicilloyl amides (Bundgaard 1976b; Yamana et al 1975b).

The structure of the side-chains in penicillins is known to have only a very small influence upon the reactivity of the β-lactam moiety in reactions with amines and other nucleophiles (Bundgaard 1976b; Schwartz 1977). This also applies to the reactivity with proteins under formation of antigenic penicilloyl-protein conjugates (Bundgaard 1977) but as demonstrated in this study ampicillin and related penicillins containing a side-chain amino group may form a notable exception. Due to the competing piperazinedione formation the efficiency of ampicillin in binding covalently to protein is much reduced as determined under conditions simulating those prevailing in vivo. Thus it can be seen from Fig. 3 that at physiological pH the penicilloylating capacity of ampicillin is greatly depressed and more than half of the protein-reacting ampicillin is transformed to piperazinedione at the expense of penicilloyl-protein conjugate. Comparing the k_1' values for ampicillin and benzylpenicillin also shows that the reactivity of ampicillin with respect to forming penicilloyl-protein conjugates at physiological pH is less than half of that of benzylpenicillin. Preliminary experiments have shown that a similar reduced tendency to bind irreversibly to serum albumin is exhibited by other amino-penicillins such as amoxycillin and cyclacillin.

It is finally worth mentioning that Batchelor et al (1965) several years ago found that ampicillin was bound to serum proteins in the form of covalent penicilloyl-protein conjugates to a markedly smaller extent than several other penicillins upon incubation with 10% human serum in a phosphate buffer at 37 °C for 48 h. This finding is in harmony with the results of the present study but it should be added that the phosphate buffer used (its concentration was not stated) may be a partly responsible factor since monohydrogen phosphate ions effectively catalyse the transformation of ampicillin to its piperazinedione derivative via a penicilloyl phosphate intermediate (Bundgaard & Hansen 1981). In separate experiments we have found that the extent of protein penicilloylation by reacting ampicillin with BSA was markedly diminished in the presence of 0·1 M phosphate buffer at pH 7·4, the piperazinedione pathway being correspondingly increased.

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