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Research Papers

PIPERAZINEDIONE FORMATION FROM REACTION OF AMPICILLIN WITH CARBOHYDRATES AND ALCOHOLS IN AQUEOUS SOLUTION

HANS BUNDGAARD and CLAUS LARSEN

The Royal Danish School of Pharmacy, Department of Pharmaceutics, 2 Universitetsparken, DK-2100 Copenhagen (Denmark)

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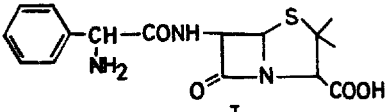
SUMMARY

The accelerating effect of various carbohydrates (e.g. glucose and dextrans) and polyhydric alcohols (e.g. sorbitol) on the rate of degradation of ampicillin in weakly alkaline solutions, has been shown to be due to a nucleophilic reaction mechanism involving formation of penicilloyl esters which subsequently undergo intramolecular aminolysis to yield a piperazine-2,5-dione derivative and, to a much smaller extent, hydrolysis to yield α-aminobenzylpenicilloic acid. Spectrophotometric and high-performance liquid chromatographic methods have been developed for the determination of the piperazinedione compound and a facile procedure to obtain this new degradation product of ampicillin in large quantities is described.

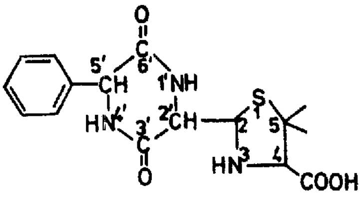
INTRODUCTION

Penicillins are often dispensed or administered in solutions containing various carbohydrates or polyhydric alcohols. Such hydroxy compounds accelerate the degradation of penicillins at neutral and alkaline pH and the kinetics of these reactions of benzylpenicillin and ampicillin have recently been the subject of various studies (Landersjö et al., 1977; Stjernström et al., 1978; Bundgaard and Larsen, 1978a, b). It has been demonstrated that the accelerated degradation of benzylpenicillin in aqueous solution caused by various carbohydrates and polyhydric alcohols (e.g. sorbitol) proceeds entirely through a nucleophilic pathway with the formation of penicilloyl esters which subsequently undergo hydrolysis to produce benzylpenicilloic acid (Bundgaard and Larsen, 1978a, b; Larsen and Bundgaard, 1978).

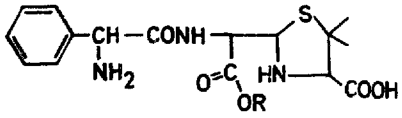
The present paper reports that although the rate effects of the polyols on the degradation of ampicillin (I) are due to a similar nucleophilic mechanism, the initial reaction product, α-aminobenzylpenicilloyl ester (II), undergoes an intramolecular aminolysis by the side-chain amino group to produce a stable piperazine-2,5-dione derivative (IV) besides undergoing, and this to a much smaller extent, simple hydrolysis into the corresponding pencilloic acid (III). In addition, a convenient and facile procedure to obtain the piperazinedione derivative in good yields is described.



I



IV



II

III R = H

MATERIALS AND METHODS

Chemicals

The ampicillin sodium was of pharmacopoeial quality (Ph. Nord. 63). Carbohydrates and other chemicals used were of reagent grade or the best possible quality. Dextran 40 and dextran 70 with average molecular weights of 41,000 and 64,400, respectively, were purchased from Pharmacia, Sweden.

General procedures

Ultraviolet spectral measurements were performed with a Perkin–Elmer 124 spectrophotometer and a Zeiss PMQ II spectrophotometer, using 1 cm quartz cells. Infrared spectra were determined on a Unicam SP 200 spectrophotometer and PMR-spectra were recorded on a JEOL C-60-H2 instrument using tetramethylsilane as an internal standard. High-performance liquid chromatography (HPLC) was done with a Spectra-Physics model 3500 B instrument equipped with a 10 μl loop injection valve. The column used, 10 cm long and 4.7 mm i.d., was packed with LiChrosorb RP-8 (5 μm particles). Melting points were determined by the capillary method and are uncorrected. Elemental analysis was carried out by P. Hansen, Microanalytical Department of Chemical Laboratory II, University of Copenhagen. The pH measurements were made at the temperature of the study using a Radiometer Type PHM 26 instrument.

Analytical procedures

The concentrations of penicilloic acid, penicilloyl ester and the piperazine-2,5-dione derivative in the reaction solutions were determined on basis of the spectrophotometric penamaldate assay (Schneider and de Weck, 1966; Schwartz and Delduce, 1969). This assay (treatment of aliquot portions of the reaction solution at pH 7.0 (0.1 M phosphate

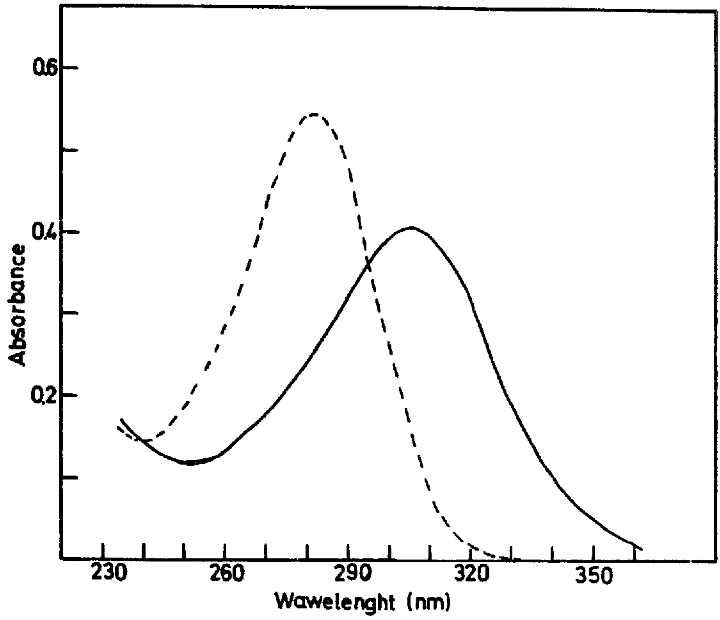


Fig. 1. The ultraviolet absorption spectra of N-(α-aminobenzylpenicilloyl)glycine (– – –) and the piperazine-2,5-dione IV (——) in 0.1 M phosphate buffer pH 7.0 after treatment with mercury(II)-chloride (penamaldate spectra). The concentrations of the compounds were 2.5×10^{-5} M.

buffer solution) with mercury(II)chloride and subsequent measurement of absorbance and absorbance-stability at 282 nm) is specific to penicilloic acids and penicilloyl derivatives (esters or amides) and permits distinction between them. Whereas the method in the form described by Schwartz and Delduce (1969) was highly useful for the determination of bezylpenicilloic acid and benzylpenicilloyl esters formed in the benzylpenicillin–carbohydrate reactions as previously described (Bundgaard and Larsen, 1978a, b; Larsen and Bundgaard 1978), it could not be used directly for the ampicillin reactions because of the similar penamaldate stabilities (94–96%) of the piperazinedione product and penicilloyl ester of ampicillin (penamaldate stability: the penamaldate absorbance value measured at 10 min from the time of addition of mercury(II)chloride in relation to the absorbance measured at time 0). However, as can be seen from Fig. 1, the penamaldate absorption spectra of the piperazinedione derivative and an α-aminobenzylpenicilloylamide (which is considered equivalent to a penicilloyl ester) are distinct with λ_{max} differing 23 nm and, therefore, it may be possible to determine the compounds in admixture by measuring the absorbances at two different wavelengths (282 and 305 nm). The following assay procedure was worked out: penicilloic acid (III) was determined in the usual manner from the recorded penamaldate absorbance–time curves (Schwartz and Delduce, 1969). Twenty minutes after the addition of mercury(II)chloride, at which time all the penamaldate derived from penicilloic acid had disappeared, the absorbance of the assay solution was determined at both 282 and 305 nm. The molar concentrations of the piperazinedione derivative (IV) and ester (II) were calculated from the following two equations:

$$A_{282} = \epsilon_{282}^{II}\,[II] + \epsilon_{282}^{IV}\,[IV] \tag{1}$$

$$A_{305} = \epsilon_{305}^{II}\,[II] + \epsilon_{305}^{IV}\,[IV] \tag{2}$$

A_{282} and A_{305} are the measured absorbances of the penamaldate assay solution at 282 and 305 nm, respectively, after standing for 20 min at room temperature, and ϵ^{II} and ϵ^{IV} represent the molar absorptivities of the compounds II and IV in the penamaldate assay at the indicated wavelengths, also taken after 20 min. The following ϵ values were determined and used in the calculations: $\epsilon_{282}^{II} = 19.0 \times 10^3$, $\epsilon_{305}^{II} = 5.7 \times 10^3$, $\epsilon_{282}^{IV} = 9.6 \times 10^3$ and $\epsilon_{305}^{IV} = 16.0 \times 10^3$ $dm^3\ mol^{-1}\ cm^{-1}$. As a model for the ester II in these calculations N-(α-aminobenzylpenicilloyl)glycine was used. This compound was prepared as previously described (Bundgaard, 1976b).

Kinetic measurements

Reaction solutions containing ampicillin sodium ($1-1.5 \times 10^{-3}$ M) and the polyol (10% w/v) in a 0.2 M aqueous carbonate buffer solution (pH 9.40) were kept at 22 ± 0.1°C. From one part of the solutions samples of 500 μl were taken at appropriate intervals and analyzed for penicilloic acid, piperazinedione derivative and ester as described above. Another part of the solutions was placed in a spectrophotometer cuvette and the decrease in absorbance at 237 nm was recorded continuously. Pseudo-first-order rate constants for the degradation of ampicillin were calculated from the absorbance-time curves by the method of Guggenheim (1926). This direct spectrophotometric assay has previously been used to measure rates of ampicillin degradation in carbohydrate solutions (Bundgaard and Larsen, 1978a, b).

RESULTS AND DISCUSSION

Isolation and identification of the piperazinedione compound (IV)

A solution of 2.5 g of ampicillin sodium in 50 ml of a 10% w/v aqueous solution of glucose with pH adjusted to 9.2 was kept at room temperature (22°C) for 20 h. The pH was maintained at 9.2 ± 0.2 by occasional addition of 2 M sodium hyroxide. Under these conditions the half-life for ampicillin was about 1.5 h and adjustment of pH was only necessary during the first 5–6 h. After completion of the reaction the solution was cooled to about 4°C and acidified to pH 2.0 with 5 M hydrochloric acid. The white precipitate formed was filtered, washed with water, air-dried, and finally recrystallized from 80% ethanol to give 1.1 g (48%) of crystalline 2-(6′-phenylpiperazin-2′,5′-dion-3′yl)5,5-dimethylthiazolidine-4-carboxylic acid (IV), m.p. 211–212°C; IR (KBr) γ_{max} 3340, 3200–2900, 1730, 1670, 1480, 1370, 1270, 1210 and 1130 cm^{-1}; PMR ($(CD_3)_2SO$) δ 1.2 (s, 3H), 1.5 (s, 3H), 3.6 (s, 1H), 3.8 (bs, 1H), 4.9 (s, 1H), 5.1 (d, J = 4 Hz, 1H), 7.4 (m, 5H), 7.8 (d, J = 3Hz, 1H) and 8.6 (d, J = 2Hz, 1H) ppm. On addition of D_2O the doublets at 7.8 and 8.6 ppm disappeared (piperazinedione amido protons (Kopple and Ohnishi, 1969; Indelicato et al., 1974, 1977)).

Analysis. Calculated for $C_{16}H_{19}N_3O_4S$: C, 55.00; H, 5.48; N, 12.03%. Found C, 54.25; H, 5.63; N, 11.67%.

The spectral and analytical data of the isolated product show this to be the piperazine-2,5-dione IV. The IR and PMR spectral data correspond well with those reported by Roets et al. (1973) for a similar piperazine-2,5-dione derivative obtained from spontaneous decomposition of 6-epi-ampicillin in neutral aqueous solution. The melting point of the latter piperazinedione is about 20°C lower than that of the former, but a difference is to be expected since the compounds have different configurations at C-2′.

The compound IV behaves analogous to penicilloyl amides and penicilloyl esters by treatment with mercury(II)chloride in neutral aqueous solution in that a strong and relatively stable absorption band with maximum at 305 nm (ϵ 17.3 × 10^3) immediately appears (Fig. 1). On acidification of this solution to pH 2 the absorption band disappeared rapidly, a fact that is characteristic of penicilloyl derivatives also (Woodward et al., 1949). Further confirmation of the proposed structure of the isolated product was obtained by analysis for primary amino groups (Bundgaard and Larsen, 1977): no reaction with the trinitrobenzenesulphonic acid reagent was observed for the product under conditions where ampicillin reacted in a concentration 30 times lower.

The piperazinedione IV could also be obtained in good yields from reaction solutions of other carbohydrates such as sucrose, fructose and dextran 40 and 70 under the same reaction conditions as described above for glucose. The same was found for reactions with alditols such as sorbitol, mannitol and glycerol.

Since this method of obtaining the ampicillin piperazinedione derivative IV appears to be a very simple procedure suitable for preparative purposes, the effect of pH on the yield was studied with glucose as reactant. From a previous study (Bundgaard and Larsen, 1978b) it is known that increasing pH speeds up the reaction. The yield of compound IV was, however, found to decrease with increasing pH at $\text{pH} > 9.5$. At lower pH values the yield remained pH-independent and, therefore, a pH of about 9–9.5 is optimal as regards yield and time of reaction. The explanation of the decreasing yield at higher pH values could be that the hydroxide ion-catalyzed hydrolysis of ester becomes increasingly predominant in relation to the spontaneous cyclization of ester to compound IV (see later).

Kinetics and mechanism of the reactions

Reaction solutions containing ampicillin sodium (1–1.5 × 10^{-3} M) and glucose or sorbitol (10% w/v) in 0.2 M aqueous carbonate buffer solutions at pH 9.40 were kept at constant temperature (22°C) and subjected to penamaldate analysis at various times as described in the experimental section. This analysis revealed the formation of penicilloic acid (III) (penamaldate stability: 20–25%, cf. Bundgaard and Larsen, 1978a) as well as penicilloyl ester (II) and the piperazinedione IV (penamaldate stability: 95%). When the absorption spectra of the penamaldate assay solutions were taken after standing for 20 min where the penamaldate deriving from the penicilloic acid had disappeared they showed a maximum at 282 nm at the beginning of the ampicillin degradation. During the reaction progress this maximum shifted to higher wavelengths until a maximum at 305 nm was reached at the completion of the degradation (Fig. 2). The final spectrum was identical to that of the piperazinedione IV in the penamaldate assay while the spectrum at the start of the reaction resembles that of a penicilloyl amide or ester (cf. Fig. 1). This observation indicates that a penicilloyl ester is formed initially, and that the

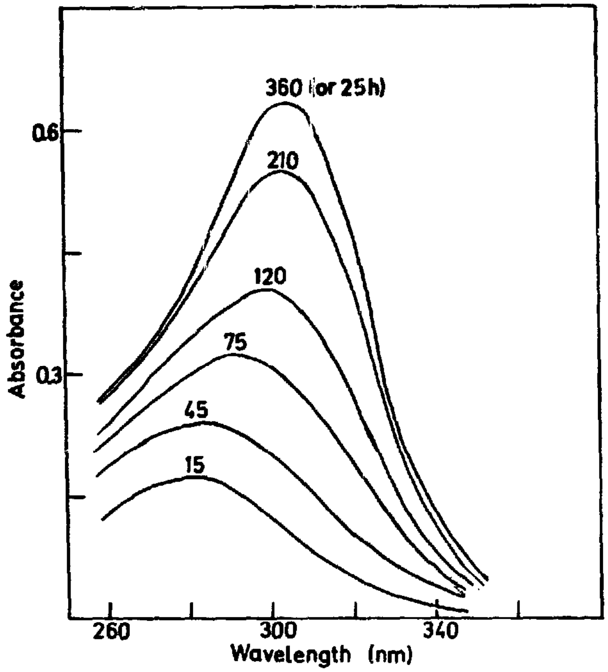


Fig. 2. Penamaldate spectra of reaction solutions of ampicillin sodium (1.6×10^{-3} M) and glucose (10% w/v) in 0.2 M aqueous carbonate buffer pH 9.40 (22°C). The curves are labelled as to minutes after start of the reaction. The spectra were taken 20 min after the addition of mercury(II)chloride and the reaction solution was diluted 31 times before being analyzed by the penamaldate assay.

piperazinedione formation takes place through this ester. When the concentrations of penicilloic acid, ester and the piperazinedione derivative were determined quantitatively during the reaction progress the time-courses shown in Figs. 3 and 4 were obtained. Included in the figures are the time-courses for the simultaneous disappearance of ampicillin as calculated from the equation:

$$\% \text{ remaining ampicillin} = 100 \times e^{-k_{obs} \cdot t} \qquad (3)$$

where the pseudo-first-order rate constant (k_{obs}) for the overall degradation of ampicillin was determined by the direct spectrophotometric method as described under Experimental. The values for k_{obs} were 0.55 h^{-1} (glucose) and 0.45 h^{-1} (sorbitol). At any time the sum of the concentrations of ampicillin and the three reaction products corresponds to 100 ± 10% in relation to the initial ampicillin concentration. As is apparent from the figures there are marked induction periods in the formation of piperazinedione and penicilloic acid. Along with the observed initial increase and subsequent decrease in ester concentration this indicates the occurrence of the consecutive reactions shown in Scheme 1. Since the penicillin is also simultaneously degraded by buffer- and hydroxide ion-catalyzed hydrolysis (Bundgaard and Larsen, 1978a, b) this reaction is included in Scheme 1. In the scheme k_1–k_4 are pseudo-first-order rate constants for the depicted

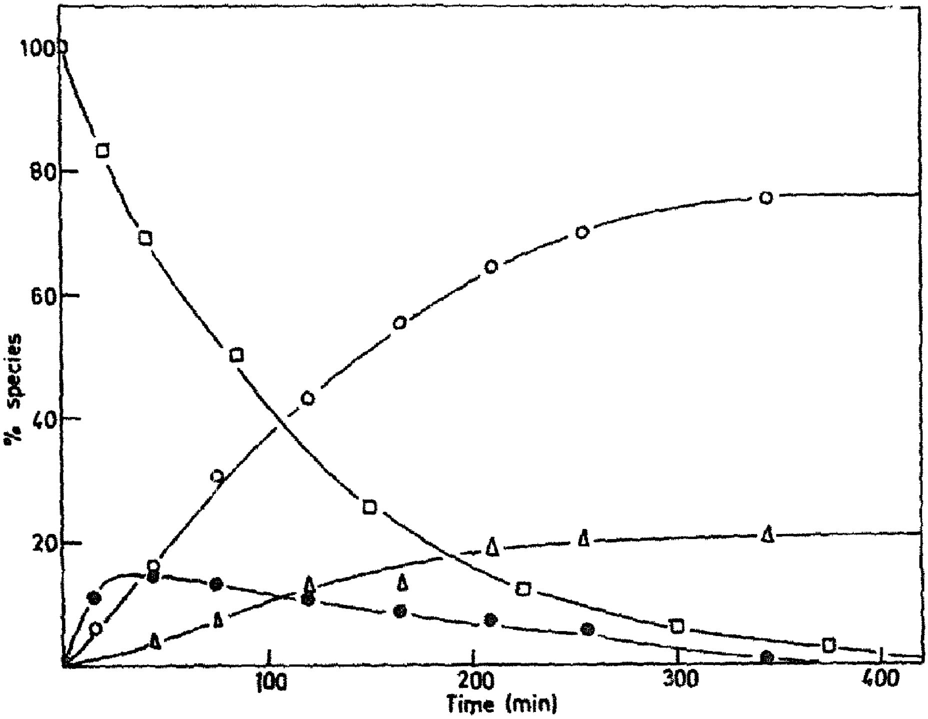
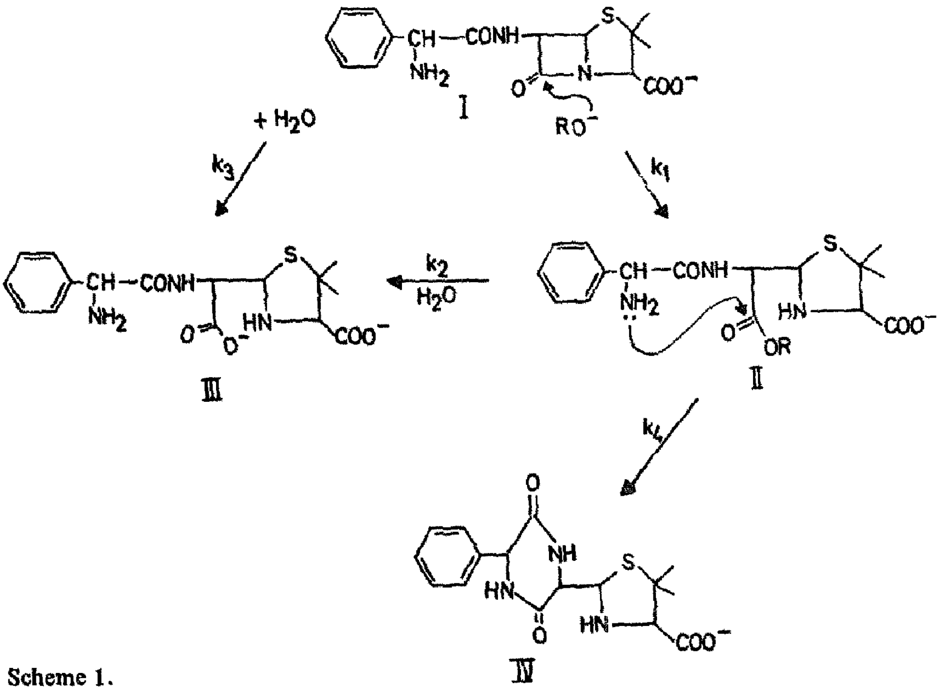


Fig. 3. Time courses for ampicillin (□), α-aminibenzylpenicilloyl glucose (●), piperazine-2,5-dione IV (○) and α-aminobenzylpenicilloic acid (△) in the reaction between ampicillin (1.55×10^{-3} M) and glucose (10% w/v) at pH 9.40 (0.2 M carbonate) and 22°C. The concentrations at various times, expressed as percent in relation to the initial penicillin concentration, were determined experimentally as described in the text.



Scheme 1.

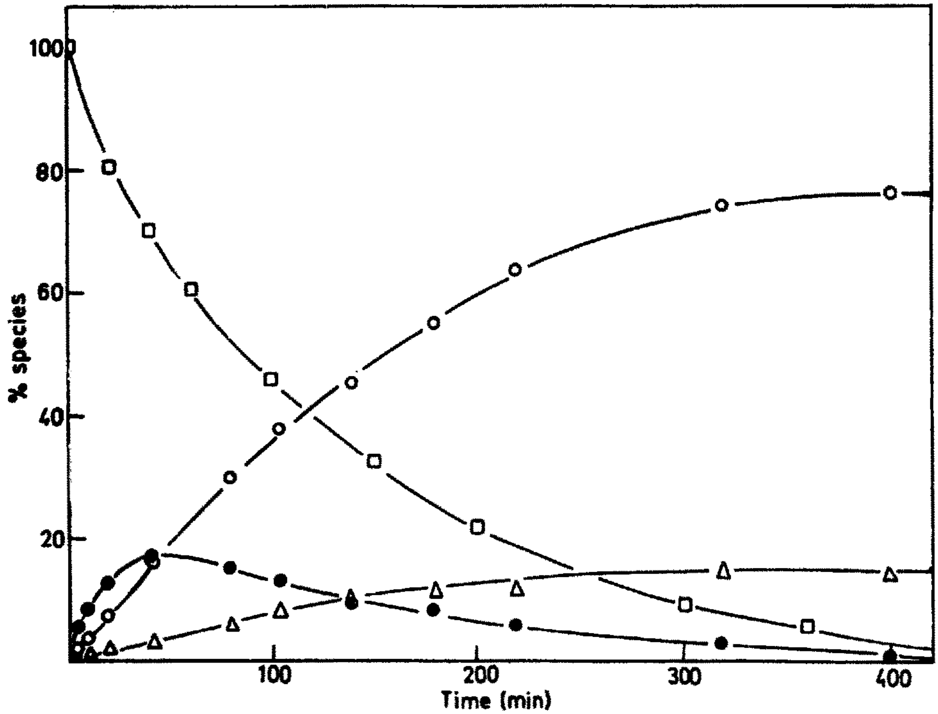


Fig. 4. Time-courses for ampicillin (□), α-aminobenzylpenicilloyl sorbitol (●), piperazine-2,5-dione IV (○) and α-aminobenzylpenicilloic acid (△) in the reaction between ampicillin (1.65×10^{-3} M) and sorbitol (10% w/v) at pH 9.40 (0.2 M carbonate) and 22°C. The concentrations at various times, expressed as percent in relation to the initial penicillin concentration, were determined experimentally as described in the text.

reactions (where $k_1 + k_3 = k_{obs}$). In the reactions with glucose and sorbitol the piperazinedione is formed in a yield of 77–80% in relation to the initial ampicillin amount with penicilloic acid accounting for the remaining about 20%. From data on reactions of benzylpenicillin under similar conditions (Bundgaard and Larsen, 1978b) the k_3 reaction can be estimated to account for about 8% of the overall ampicillin degradation. Thus, the ratio k_4/k_2 can be estimated to be $80/(20 - 8) \simeq 7$.

The simultaneous disappearance of ampicillin and appearance of the piperazinedione and penicilloic acid in the glucose reaction mixture was also observed by HPLC (Fig. 5). Using this specific method the final yields of these two reaction products were the same as when the penamaldate methods were used. Quantitation was done by relating the peak heights to those of standards chromatographed under the same conditions. The spectrophotometrically determined value of k_{obs} for ampicillin degradation was also confirmed chromatographically by following the disappearance of the ampicillin peak with time.

Considering the mechanism of the reactions we have in previous studies shown that the accelerating effect of carbohydrates and various other polyols on the rate of degradation of penicillins is entirely due to nucleophilic displacement reactions at the penicillin β-lactam carbonyl moiety by an alkoxide ion derived from proton ionization of one or more of the hydroxyl groups to produce penicilloyl esters, which then hydrolyze to yield penicilloic acid (Bundgaard, 1976a; Bundgaard and Larsen, 1978a, b; Larsen and

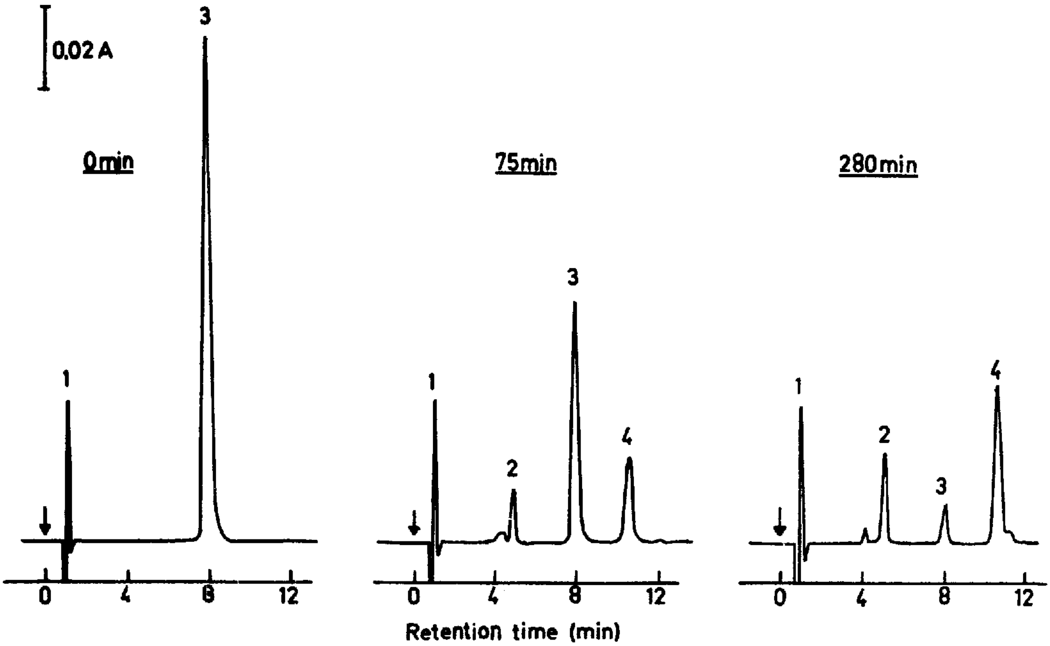
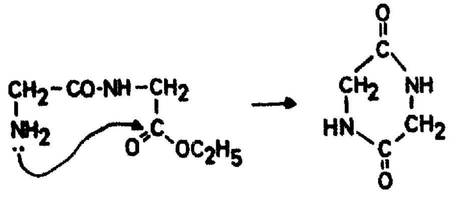


Fig. 5. High-performance liquid chromatographic changes for the degradation of ampicillin (0.017 M) in 10% w/v glucose solution of pH 9.40 (0.2 M carbonate) at 22°C with time in minutes (listed on the graph). A 10 μl sample of the solution was chromatographed. Chromatographic conditions: column, 100 × 4.7 mm i.d., with LiChrosorb RP-8 ($d_p = 5$ μm); eluent, methanol (25% w/v) and tetrabutylammonium bromide (0.01 M) in 0.01 M citrate buffer pH 6.0; flow-rate, 1.6 ml/min; temperature, 22°C; detection, UV at 240 nm, 0.2 a.u.f.s. Peak identities: 1 = solvent peak; 2 = α-aminobenzylpenicilloic acid; 3 = ampicillin; 4 = piperazine-2,5-dione IV.

Bundgaard, 1978). This study shows that when an α-amino group is present in the penicillin side-chain the penicilloyl esters undergo an intramolecular aminolysis by this side-chain amino group to produce a stable piperazine-2,5-dione derivative besides being hydrolyzed into penicilloic acid as depicted in Scheme 1. At the reaction conditions concerned the intramolecular aminolysis of the penicilloyl ester proceeds about 7 times more rapidly than hydrolysis of the ester, and the piperazinedione is consequently the dominating overall degradation product. The reacting form of the amino group is the free base, and since the pK_a is 7.25 at 25°C (Rapson and Bird, 1963) all is in this form at the weakly alkaline conditions.

Methyl and ethyl esters of various dipeptides, e.g. glycylglycine, are known to undergo facile cyclization in weakly alkaline aqueous solutions into the corresponding piperazine-2,5-diones (Meresaar and Ågren, 1968; Purdie and Benoiton, 1973) (Scheme 2), and since the penicilloyl esters from ampicillin are in fact dipeptide esters (substituted glycylglycine esters) their ready cyclization into the piperazinedione IV is quite analogous to the reactions of the simple dipeptide esters.



Scheme 2.

Immunological aspects

Penicilloate esters formed from reaction of benzylpenicillin with raffinose, inulin, carboxylmethyl cellulose and various dextrans have been reported to be capable of eliciting penicilloyl-specific allergic reactions in sensitized animals (Schneider and de Weck, 1969; Schneider et al., 1971a, b; Molinari et al., 1973), and therefore the presence of even small amounts of such or perhaps also other penicilloyl–carbohydrate conjugates in clinically used penicillin preparations may play a part in the elicitation of penicillin allergic reactions. Ampicillin is often administered by infusion of carbohydrate-containing intravenous fluids. The addition of ampicillin salts to such fluids usually gives them an alkaline pH (Stjernström et al., 1978) corresponding to the conditions used in the present study. The results presented here show that although the piperazinedione derivative constitutes the predominant degradation product in weakly alkaline carbohydrate solutions the penicilloyl carbohydrate esters are present in significant amounts in the reaction pathway and – what is important from a practical and immunological point of view – the relative concentrations of the esters are greatest in the early phase of ampicillin decomposition. Thus, it can be seen from Figs. 3 and 4 that at an ampicillin degradation corresponding to 10-20% the esters constitute the major degradation products present in the solutions. This was also found to be the case for a commercial dextran-containing infusion fluid (Rheomacrodex 10%). To this 10% solution of dextran 40 in 0.9% sodium chloride was added ampicillin sodium (1.5%), giving a pH of 9.02. After standing for 2 and 5 h at room temperature about 10 and 20%, respectively, of the ampicillin had degraded. Penamaldate analysis after the mentioned periods showed that 60–80% of the degraded ampicillin were in the form of penicilloyl dextran esters.

To reduce the rate of formation of potentially antigenic penicilloyl esters in ampicillin–carbohydrate solutions the pH should be as low as practically possible (Bundgaard and Larsen, 1978b).

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