# A Nuclear Magnetic Resonance Study of the Degradation of Penicillin G in Acidic Solution

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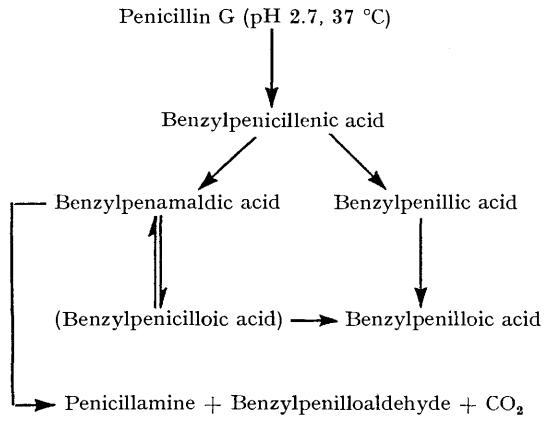
N.m.r. methods have been used to study the degradation of benzylpenicillin at pH 2.5 and 37 °C. After 100 min three major reaction products, penamaldic acid, penillic acid, and penicilloic acid were detected. By measuring the extent of deuteriation at the C-6 position (the reaction was conducted in DCl–$D_2O$ solution) it was shown that most of the penillic acid (65%) and penicilloic acid (70%) had not been formed *via* a penicillenic acid type intermediate as previously suggested. Eventually the three initial products degraded into penilloic acid without any further deuteriation (estimated from the deuteriation at the C-6 position of penilloic acid): thus penillic acid and penicilloic acid do not degrade *via* a penicillenic acid intermediate. No penicillamine, benzylpenilloaldehyde, or related products were detected.

SINCE the discovery of penicillin in 1929 [1] there have been many studies of its degradation in acidic media, with two main aims in view. First, it was hoped that an understanding of the mechanism of degradation would help in the search for new orally active penicillins which would not be degraded in the acidic conditions prevailing in the stomach. Secondly, it was thought that such studies might lead to an understanding of allergenic responses to penicillin which might be related to the formation of complexes between proteins and penicillin degradation products. These problems have been extensively reviewed in two recent articles.[2,3]

In spite of these extensive studies there is still considerable uncertainty about the details of the reaction pathway. In the early work on penicillin degradation it was established that penillic acid is a major product formed in acid media.[4] Later, Dennen and Davis [5] proposed that penicilloic acid is also an important degradation product. From a consideration of the results of Krejci,[6] Schwartz [7] has suggested that one reaction pathway involves acid catalysed hydrolysis of the undissociated penicillin molecule leading to penilloic acid while in a second parallel reaction penicillin forms penicillenic acid which rearranges to penillic acid. Other workers,[8,9] have postulated that an oxazolone-thiazoline intermediate exists on the formation pathway of penicillenic acid, penicillamine, and penaldic acid.

The most recent study of the mechanism of acidic degradation of penicillin is that reported by Blaha [10,11] who proposes the following detailed scheme:




Benzylpenicillenic acid, which is easily detected by its intense u.v. absorption at 322 nm is formed as a short lived intermediate.[10,11] It is formed in the early stages of the reaction and reached its maximum concentration after a few minutes. Blaha [10,11] has proposed that this key intermediate is on the main formation pathway of all the other main degradation products as indicated above.

In the present paper we report n.m.r. measurements on the degradation products of penicillin at pH 2.5 and 37 °C which clearly show that, while penicillenic acid is an intermediate in the formation of some penillic and penicilloic acids, it is not on their major formation pathways. Our findings allow us to propose a modified scheme for the mechanism of penicillin degradation in acidic media.

## EXPERIMENTAL

Solutions of penicillin G ($5 \times 10^{-3}$—$5 \times 10^{-2}$M) in DCl–$D_2O$ were adjusted to pH 2.5 and allowed to degrade at 37 °C. The pH was readjusted during the degradation process such that the pH never deviated by more than 0.1 units from the initial value.

At various times, samples (0.5 ml) were extracted from the reaction vessel, transferred to n.m.r. tubes (5 mm) and their n.m.r. spectra acquired. No noticeable difference was observed in the nature of the degradation products from solutions with different penicillin concentrations in the range $5 \times 10^{-3}$—$5 \times 10^{-2}$M. For this reason most of the experiments were performed at the higher concentration ($5 \times 10^{-2}$M) for which good signal-to-noise spectra could be obtained in a very short time.

N.m.r. spectra were obtained using a Brüker WH270 Fourier transform spectrometer equipped with a variable temperature accessory. 8192 Data points were used in acquiring spectra with widths of 4 000 Hz. For most samples 250 transients were accumulated. Some experiments were carried out using pulse intervals of 5 s to give more accurate intensity measurements. The pH measurements were made using a Radiometer model 26 pH meter with a combination glass electrode: the pH values quoted are all meter readings uncorrected for deuterium isotope effects.

U.v. spectra were recorded using a Pye–Unicam SP 1800 spectrophotometer.

*Materials.*—Penicillin G (sodium salt), benzylpenicillenic acid, and D-penicillamine were purchased from Sigma

Chemicals and used without further purification. Penilloaldehyde, thiazoline, *N*-formyl-D-penicillamine, phenylacetylglycine, benzylpenillic acid, benzylpenilloic acid, and benzylpenicilloic acid were synthesised using standard methods.[4]

RESULTS AND DISCUSSION

The $^1$H n.m.r. spectrum of the degradation products of penicillin G formed after 100 min at pH 2.5 and 37 °C is shown in Figure 1. The chemical shifts and assignments of the signals are given in the Table. Most of the signal assignments were made by comparing the chemical shifts with those from standard compounds examined under the same conditions (see Table). The standard compounds used were penillic, penicillenic, penicilloic, and penilloic acids, penilloaldehyde, thiazoline, D-penicillamine, *N*-formyl-D-penicillamine, and phenylacetylglycine. The n.m.r. data for penamaldic acid were obtained from studies of the acid degradation of penicillenic acid which has been shown by Longridge and his co-workers [12] to form penamaldic acid and penillic acid in the pH range 2.0—2.9. In the n.m.r. spectra of the degradation products of penicillenic acid (at pH 2.5, 37 °C, and with *ca.* 2% acetone present to improve solubility) we detected approximately equimolar amounts of penamaldic acid and penillic acid.

*Kinetic Data.*—In the early stages of the reaction n.m.r. signals were detected from three products, namely penillic, penicilloic, and penamaldic acid. These products appear almost simultaneously in the reaction mixture. Penicillenic acid, which we confirmed is present from the u.v. absorption at 322 nm, is not detected in the n.m.r. spectrum presumably because of its low concentration ($<10^{-4}$M). After 100 min almost all the penicillin G has disappeared (see Figure 1) and the relative amounts of the products at this stage are 57% (penillic acid), 24% (penamaldic acid), and 19% (penicilloic acid). After one day the solution gives an n.m.r. spectrum containing extra lines corresponding to penilloic acid formed, at this stage, mainly by decarboxylation of penicilloic acid. As the degradation proceeds, the penilloic acid continues to increase in concentration

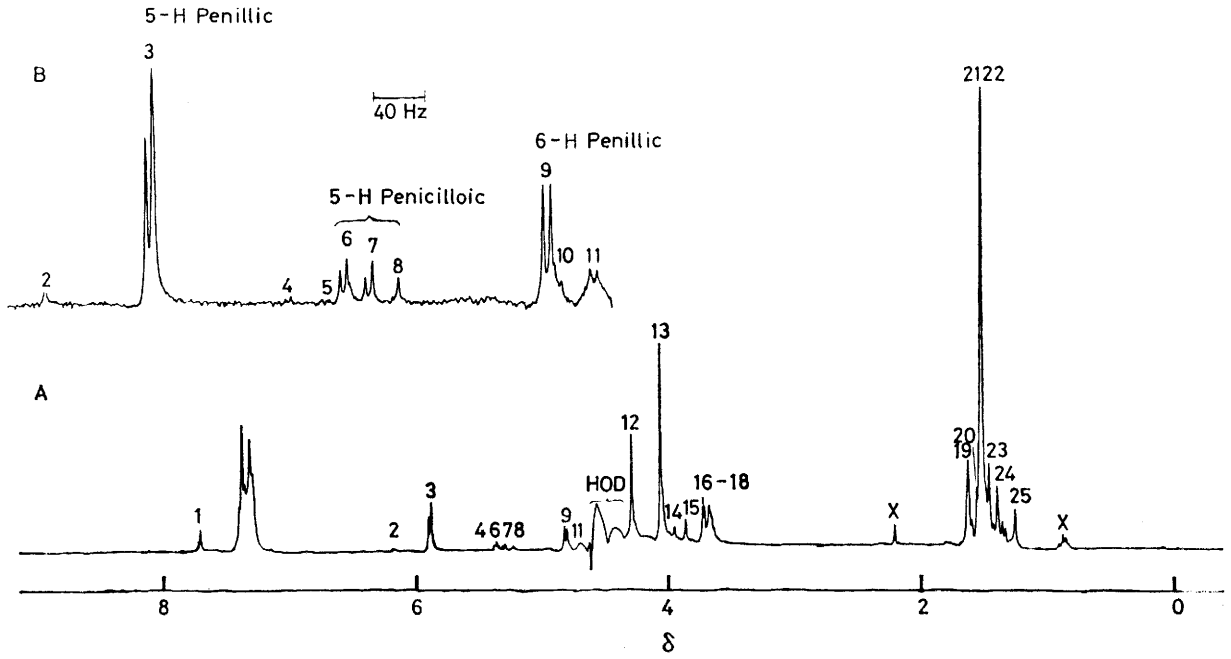



FIGURE 1 A, The $^1$H 270 MHz n.m.r. spectrum of the acid degradation products of benzylpenicillin at pH 2.5 and 37 °C after 100 min. The assignment of the bands is given in the Table (×, peaks from impurities); B, expanded region of spectrum

as the other products decrease. After 10 days all the penamaldic acid has disappeared and penillic acid has

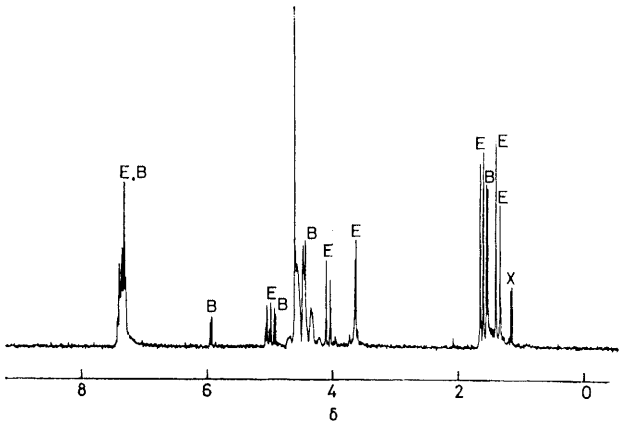



FIGURE 2 The $^1$H 270 MHz n.m.r. spectrum of the acid degradation products of benzylpenicillin at pH 2.5 and 37 °C after 10 days (×, impurity): B, penillic acid; E, penilloic acid

decreased to 25% of the total concentration (see Figures 2 and 3). After 30 days all the intermediate products have degraded into penilloic acid. This is consistent with our results on the acid degradation of the pure

compounds, penillic and penicilloic acid, which yield penilloic acid in each case.

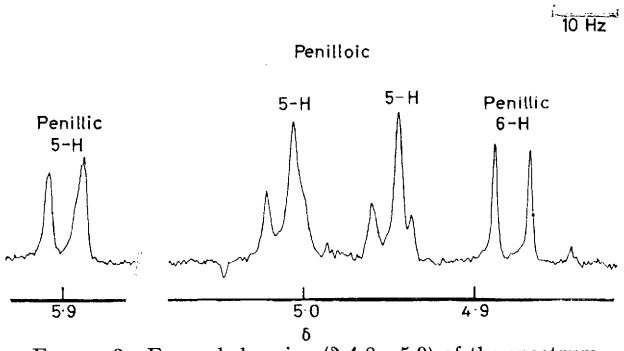



FIGURE 3 Expanded region (δ 4.8—5.9) of the spectrum shown in Figure 2

The relative concentrations of the various products at different stages of the reaction can be measured from the integrated intensities of their n.m.r. signals. It was thus possible to follow the changes in concentrations of the products as a function of time and obtain kinetic data as shown in Figures 4 and 5. The apparent first-order rate constant for the loss of penicillin G is similar to that found by Blaha (0.44 $min^{-1}$ in this study and 0.29 $min^{-1}$ in Blaha's study). The small difference could be due to their somewhat different conditions [Blaha worked on a 0.005M-potassium benzylpenicillin solution at pH 2.7 in citric acid–disodium phosphate buffer (0.003 7M)]. We also followed the rates of appearance and disappearance of penicillenic acid by u.v. absorption at 322 nm; they were identical to those found by Blaha.[10,11] However, in contrast to her results, we were unable to detect either D-penicillamine or penilloaldehyde even in solutions examined after 10 days. Furthermore we could easily detect large amounts of penicilloic acid (*ca.* 19% after 100 min) which had proved difficult to detect by the high pressure liquid chromatographic–u.v. methods employed by Blaha.[11]

It is worth noting that although the 5*R*,6*R*-diastereoisomer of penicilloic acid is formed at the early stage of the reaction, after 100 mins a mixture of 5*R*,6*R*- and 5*S*,6*R*-diastereoisomers is obtained. This is to be expected because (5*R*,6*R*)-penicilloic acid epimerises to a 5*R*,6*R*–5*S*,6*R* mixture at pH 2.5 and 37°. Penilloic acid, which is the dominant product after 10 days, also exists as a similar mixture of diastereoisomers.

The $^1H$ chemical shifts [a] of the degradation products of benzylpenicillin after 100 min at pH 2.5 and 37 °C and of some related reference compounds

| Line [b] number | Penicillin G degradation products | Penicillin G | Penillic acid | Penicilloic acid [c] | Penamaldic acid |
|---|---|---|---|---|---|
| 1 | 7.78 | | | | 7.78 (5-H) |
| 2 | 6.22 [d] | | | | |
| 3 | 5.94 | | 5.90 (5-H) | | |
| 4 | 5.56 | 5.53 (6-H) | | | |
| 5 | 5.45 | 5.45 (5-H) | | | |
| 6 | 5.40 | | | 5.38 (*RR*) (5-H) | |
| 7 | 5.33 | | | 5.33 (*SR*) (5-H) | |
| 8 | 5.25 [e] | | | | |
| 9 | 4.84 | | 4.84 (6-H) | | |
| 10 | 4.81 | | | 4.82 (*SR*) (6-H) | |
| 11 | 4.72 | | | 4.72 (*RR*) (6-H) | |
| 12 | 4.32 | | 4.30 (3-H) | | |
| 13 | 4.09 | | 4.07 (9-H) | 4.08 (*SR*) (3-H) | |
| 14 | 3.98 | | | 3.95 (*RR*) (3-H) | |
| 15 | 3.88 | | | | 3.90 (3-H) |
| 16 | 3.75 | 3.77 (9-H) | | | |
| 17 | 3.72 | | | | 3.73 (9-H) |
| 18 | 3.69 | | | 3.70 (*SR*) (9-H) | |
| | | | | 3.68 (*RR*) (9-H) | |
| 19 | 1.64 | | | 1.62 (*SR*) (Me) | 1.62 (Me) |
| 20 | 1.57 | 1.56 (Me) | | 1.57 (*RR*) (Me) | |
| 21 | 1.54 | | 1.53 (Me) | | |
| 22 | 1.52 | 1.50 (Me) | 1.51 (Me) | | |
| 23 | 1.47 | | | | 1.45 (Me) |
| 24 | 1.40 | | | 1.39 (*RR*) (Me) | |
| 25 | 1.26 | | | 1.26 (*SR*) (Me) | |

[a] In p.p.m. from reference DSS. [b] Line numbers refer to Figure 1. [c] Mixture of 5*R*,6*R*- and 5*S*,6*R*-isomers (labelled *RR* and *SR* respectively). [d] This signal corresponds probably to 5-H of a penillic acid stereoisomer. [e] This signal corresponds probably to 5-H of the 5*R*,6*S* stereoisomer of penicilloic acid. [f] The chemical shift data for penilloic acid are 5-H (δ 4.95, 5.01), 3-H (δ 4.03, 4.10), 9-H (δ 3.60, 3.70), $CH_3$ (δ 1.35, 1.42, 1.62, 1.67).

*Reaction Pathways.*—Because of the unstable nature of penicillenic acid it has previously proved difficult

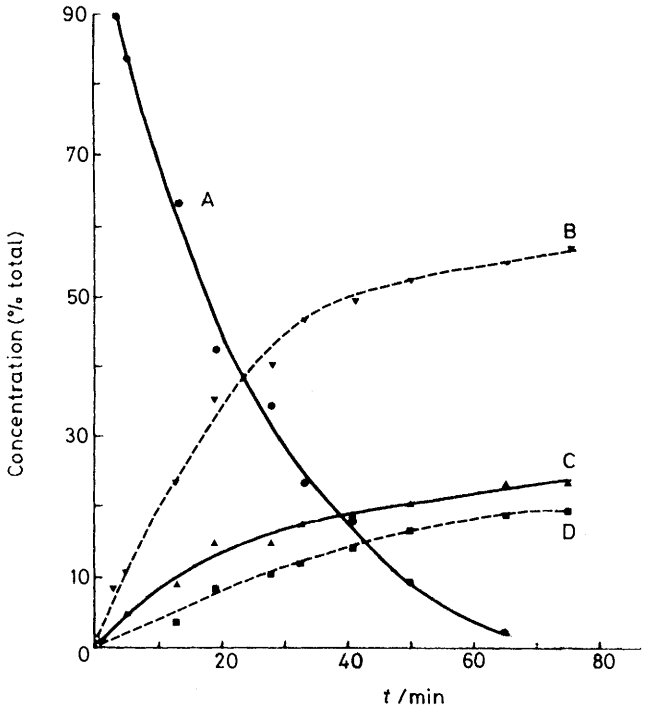



FIGURE 4 A plot of the concentrations of the degradation products of benzylpenicillin at pH 2.5 and 37 °C measured as a function of time (0—80 min): A, benzylpenicillin; B, penillic acid; C, penamaldic acid; D, penicilloic acid

to define its exact location in the reaction scheme. However, we have now found an easy method of detecting when it is not on the main degradation pathway of another intermediate. Penicillenic acid has a double bond between the C-5 and -6 and thus has no hydrogen at C-6. In the present study where the reaction is carried out in DCl–$D_2O$ solution, products which have a single bond between C-5 and C-6 and which have been formed from penicillenic acid will bear a deuterium on C-6. This deuterium can easily be detected by examining the 5- and 6-H resonances of the products. In the spectra of the standard compounds, penillic and penicilloic acid, we observe doublets for 5- and 6-H ($J_{5,6}$ 5.5 for penillic acid and 4.0 Hz for penicilloic acid). In the spectra of the compounds which bear a deuterium on C-6 we would expect the 6-H signal to be absent and the doublet for 5-H to be replaced by a broad singlet (small triplet splitting $J_{HD} = 0.15J_{HH}$). A close examination of the 5-H signals of the degradation products penillic and penicilloic acid (see Figure 1B) reveals that in addition to the well-resolved doublets from 5-H–6-H spin-coupling there are additional singlet absorptions. For penillic acid 5-H gives rise to a doublet with a singlet superimposed on the high field component of the doublet. (Due to the isotope effect on the chemical shift, one would expect that the singlets will be somewhat to high field of the centre of the doublet). The 6-H resonance gives rise to a symmetrical doublet with an intensity substantially less than that of the 5-H resonance due to deuteriation at C-6 (the absence of a singlet absorption in the 6-H signal proves that there is no deuteriation at C-5). Measurement of the relative intensities of the 5- and 6-H signals indicates that 35% of the penillic acid has been deuteriated at C-6. Thus the remaining 65% of penillic acid cannot have been formed from penicillenic acid (or any related compound having a 5,6-double bond). For penicilloic acid, the 5-H signals of the 5*R*,6*R*- and 5*S*,6*R*-diastereoisomers (see Figure 1 and Table) each comprise a singlet overlapping the high field component of a doublet: there is an additional singlet at δ 5.25 which probably corresponds to the 5*R*,6*S*-diastereoisomer. From measurement of the relative intensities of singlets and doublets of the 5-H resonances we estimate that 30% of penicilloic acid is deuteriated at C-6. Thus 70% of this product does not have a penicillenic acid type intermediate on its formation pathway. The 6-H signals of penicilloic acid (bands 10 and 11 in Figure 1B) overlap other resonances which makes it more difficult to exclude the possibility of deuteriation at C-5. However, the epimerisation of penicilloic acid at pH 2.7 in $D_2O$ solution does not lead to deuteriation at C-5.

In summary the proportions of the initial products which have an intermediate related to penicillenic acid on their formation pathway are: penamaldic acid, 100%; penillic acid, 35%; and penicilloic acid, 30%.

In the spectrum obtained after 10 days reaction (Figures 2 and 3) 6-$H_2$ of penilloic acid show two resonance bands for the two epimeric forms (5*R* and 5*S*), each absorption consisting of a doublet overlapped by a singlet band. The latter again arise from species which are deuteriated at the 6-position as expected for molecules derived from an intermediate related to penicillenic acid. Measurement of the relative intensities of the singlet and doublet absorptions of the 5-H resonances

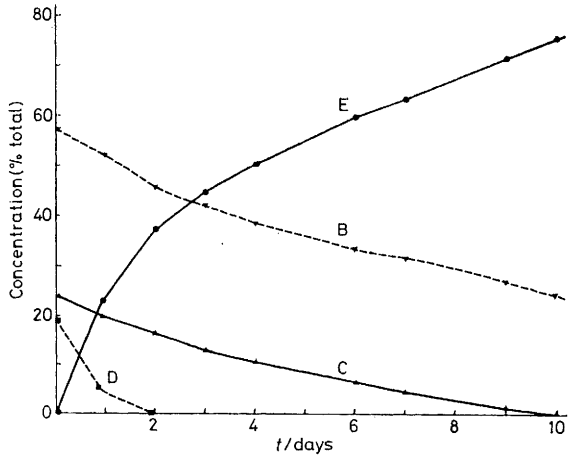
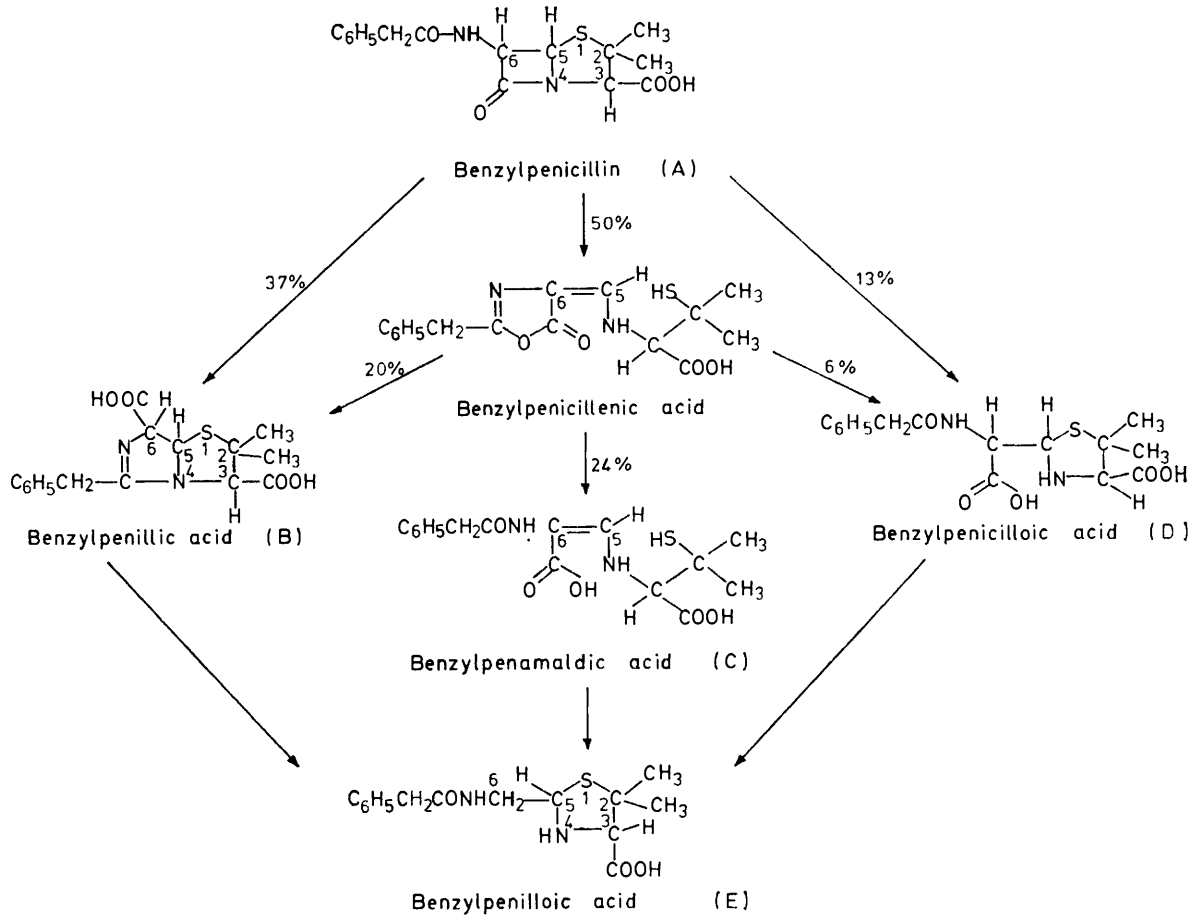



FIGURE 5 A plot of the concentrations of the degradation products of benzylpenicillin at pH 2.5 and 37 °C measured as a function of time (0—10 days): E, penilloic acid; B, penillic acid; C, penamaldic acid; D, penicilloic acid

of penilloic acid indicates that 34 ± 3% of the original penicillin has degraded to penilloic acid through such an




SCHEME

intermediate. If we assume that the products formed after 100 min (see above) degrade to penilloic acid without further deuteriation, we calculate that 38% of the original penicillin would have degraded to deuteriated penilloic acid. This reasonable agreement between observed and calculated values indicates that neither penillic acid nor penicilloic acid degrade to penilloic acid *via* a penicillenic acid type intermediate.

We could find no evidence that penicilloic and penillic acid are in reversible equilibrium with each other. Because penicilloic acid degrades to penilloic acid much more rapidly ($t_{1/2}$ *ca.* 7 h) than does penillic acid ($t_{1/2}$ *ca.* 8 days) we cannot decide whether penillic acid arrives at penilloic acid directly or *via* penicilloic acid.

Based on the above results we can propose the degradation Scheme, in which we have indicated the percentage of the original benzylpenicillin which follows each pathway (calculated from the proportions of penamaldic acid, penicilloic acid, and penillic acid formed in the first stage of the reaction and from the deuteriation experiments).

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