Inhibition of Penicillin Biosynthesis by δ-(L-α-Amino-δ-adipyl)-L-cysteinylglycine. Evidence for Initial β-Lactam Ring Formation

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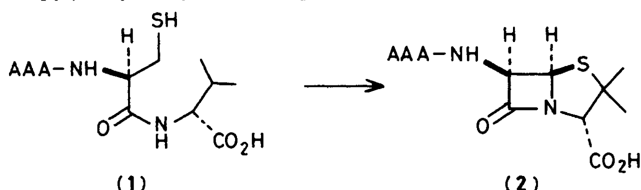
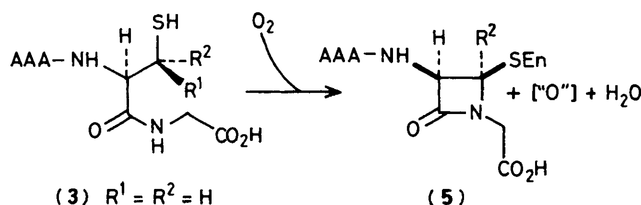
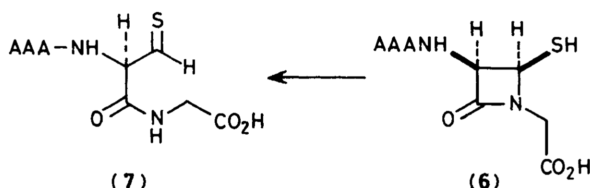
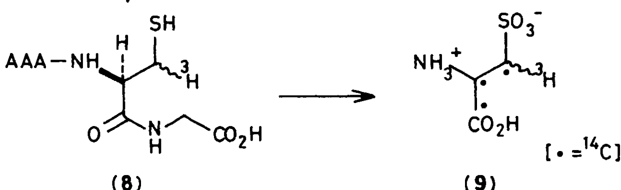
Incubation of the modified substrate, δ-(L-α-amino-δ-adipyl)-L-cysteinylglycine (ACG) with the enzyme isopenicillin N synthetase resulted in inhibition at a rate which paralleled the rate of release of tritium (as ^3HOH) from the [3-^{3}H]cysteinyl isotopomer of ACG, while incubation of the [U-^{14}C]cysteinyl isotopomer of ACG with the enzyme, in the presence of NaB^3H_4, gave rise to ^{3}H-labelled ACG, in which the tritium was shown to reside at the 3-cysteinyl position; these results are in accord with β-lactam formation during enzymic transformation of this substrate.

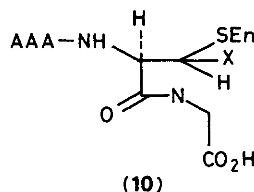
During studies on the enzymic conversion of δ-(L-α-amino-δ-adipyl)-L-cysteinyl-D-valine [ACV, (**1**)] into isopenicillin N (**2**) we found evidence that formation of the thiazolidine ring of penicillins by the purified enzyme, isopenicillin N synthetase, probably proceeds *via* homolytic pathways.[1] Since this enzyme is an iron- and dioxygen-dependent dehydrogenase[2] it appeared possible that if the reaction proceeded by initial β-lactam formation, as was envisaged,[1] then the title tripeptide, ACG (**3**), being structurally unable to provide a thiazolidine ring, might employ the unused oxidising equivalent {as ["O"] in Scheme 1}, in a suicide inhibition pathway, from the enzyme bound β-lactam intermediate (**5**), Scheme 1. Furthermore were this β-lactam (**5**) to be removed from the inactivated enzyme it could be specifically labelled, since previous experience in our laboratory has shown that these 4-mercaptoazetidinone-containing peptides (**6**) can be deuteriated as (**8**), by direct reduction with NaB^2H_4 *via* the thioaldehyde (**7**).[3] Consequently we have undertaken experiments to ascertain whether tritium is released from (**4**) during inactivation of the enzyme and whether it can then be reinserted into (**3**) by way of Scheme 1.











Scheme 1. AAA = δ-(L-α-amino-δ-adipyl) = 5-(5*S*)-amino-5-carboxypentanoyl, En = enzyme.

Incubation for 30 min under standard conditions† of δ-(L-α-amino-δ-adipyl)-L-[3-^{3}H]cysteinyl[-^{14}C]glycine[4] (**4**) with purified isopenicillin N synthetase[5] followed by denaturation with the detergent sodium dodecyl sulphate (SDS) and distillation gave tritiated water, the amount of tritium released being approximately proportional to the enzyme concentration, Figure 1.‡

In control experiments with denatured enzyme (SDS, urea) the tritium released was only some 9% of that with the native enzyme, indicating that the production of tritiated water was indeed consequent on the enzymic activity. The time dependence of both tritium release and of inactivation of the synthetase by ACG was determined under the same conditions. The results, shown in Figure 2, indicate that both processes occur at very similar rates, with a linear correlation (r) greater than 0.99.§

Interception of the presumed β-lactam thiol (**6**) was

† These were: enzyme (2.2 Iu, in Tris Buffer, 560 μl), peptide (3.7 mM, aqueous, 150 μl), ascorbate (50 mM, 50 μl), ferrous sulphate (5 mM, 50 μl), catalase (Sigma, 10%, 7 μl), and dithiothreitol (100 mM, 24 μl) to pH 7.5 and incubated for 30 min at 37 °C.

‡ As a result of difficulties in determination of the precise enzyme molarity we cannot, at this stage, give the numerical stoicheiometry of the tritium release *vs.* enzyme molarity. Similarly we cannot yet determine the stoicheiometry of tritium insertion *vs.* enzyme molarity.

§ Control experiments conducted with denatured (SDS, urea) enzyme under these time conditions showed a background of *ca.* 9% release of tritium from the peptide after 150 min. There seems to be some non-enzymic autoxidation pathway responsible for this. The curves of Figure 2 have been corrected for this. Enzyme activity was determined by assay with *Staphylococcus aureus*.[5]

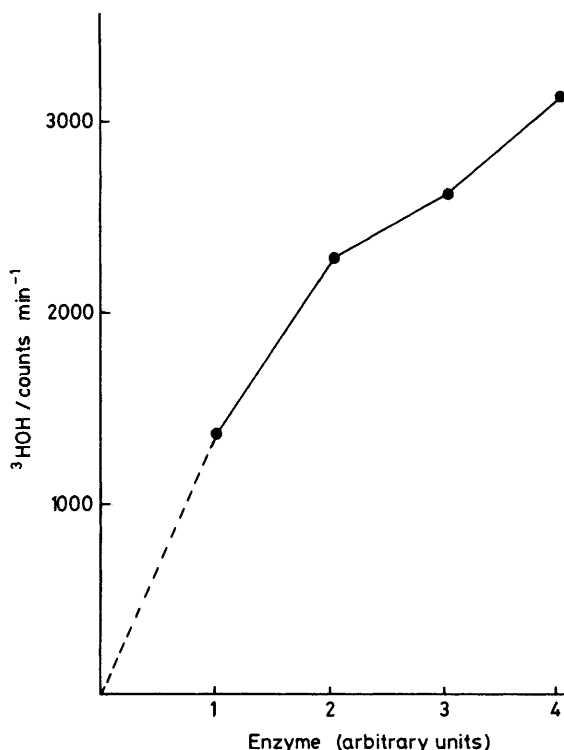


Figure 1. Tritium release as ^{3}HOH from (**4**) as a function of enzyme concentration.

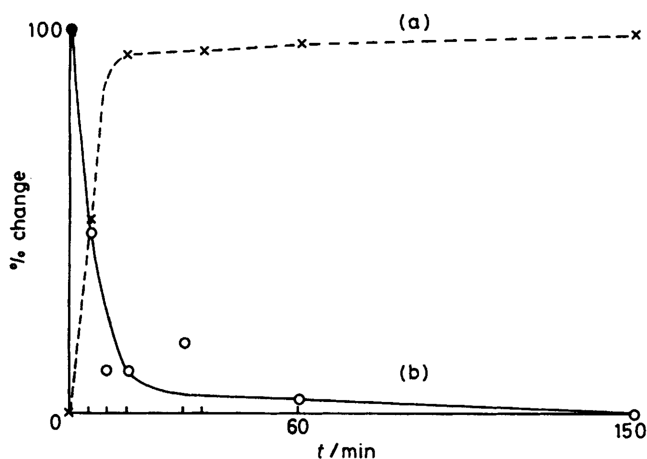


Figure 2. The time dependence of (a) ^{3}HOH release from (**4**) and (b) inactivation of isopenicillin N synthetase.

achieved by incubation of [U-^{14}C]cysteine-derived ACG, essentially as above, but in the presence of NaB^3H_4 (250 equiv. *vs.* ACG), which was shown in bioassay not to affect the enzyme's activity. The ACG was reisolated at intervals, purified (paper electrophoresis, pH 1.8) as the derived sulphonic acid (performic acid, 1.5%), hydrolysed (6 M HCl), and repurified (paper electrophoresis, pH 1.8) as cysteic acid (**9**). The time course of incorporation of tritium into this peptide is shown in Figure 3.¶ The tritium was entirely at C-3, since it was not lost during the acid conditions of peptide hydrolysis. We have shown that (**9**) undergoes clean proton

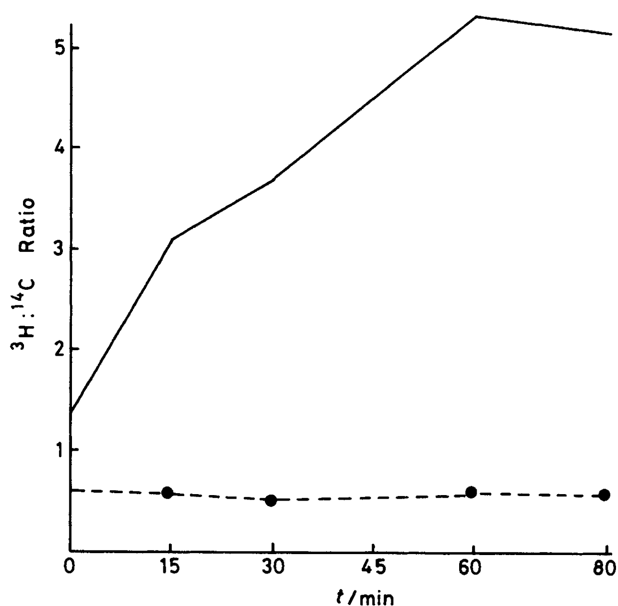


Figure 3. The rate of incorporation of ^{3}H into (**9**). —— Test, ---- control.

exchange at C-2 (D_2O–DCl experiment) under these conditions.

In summary, we have observed that inactivation of isopenicillin N synthetase by ACG occurs at virtually the same rate as does tritium release from the 3-cysteinyl position in this peptide. Also, in the presence of NaB^3H_4 we can reintroduce tritium into this site. We believe that these experiments support an inhibition mechanism which involves the formation of an enzyme bound β-lactam, as (**5**), which can in the presence of dithiothreitol (DTT) be released as (**6**) and intercepted in solution. An alternative explanation of these results, *i.e.* that an enzyme-bound thioaldehyde equivalent, as (**10**), was formed instead of a β-lactam cannot presently be eliminated from consideration. However, since β-lactam formation is part of the normal pathway, from ACV, we favour the species (**5**) as the intermediate in this inactivation process.

We thank Drs. R. M. Adlington and J. J. Usher for helpful discussions.

Received, 26th March 1984; Com. 407

¶ Because of the instability of NaB^3H_4 in solution, the incubation was initiated at 0 °C and warmed to 20 °C; therefore the rates of these incorporations are not directly comparable to those of tritium release.

References

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