# Stoichiometry of oxygen consumption in the biosynthesis of isopenicillin from a tripeptide

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The biosynthesis of isopenicillin N from δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine in a
cell-free system has been correlated with O_2 consumption by two methods, involving the
use of an oxygen-electrode and an n.m.r. spectrometer respectively. The results are
consistent with a 1:1 stoichiometric ratio for the dioxygen consumed to the isopenicillin
N formed.

A cell-free system from *Cephalosporium acremonium* catalyses the conversion of the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (1) into isopenicillin N (2) in the presence of O_2 and ferrous iron (O'Sullivan *et al.*, 1979; Konomi *et al.*, 1979; Abraham *et al.*, 1981). Measurements of the quotient (isopenicillin N formed)/(dioxygen consumed) by two methods are described in the present paper. In one method, dioxygen consumption was determined with an oxygen electrode and isopenicillin synthesis by antibacterial assay. In a second method, the formation of isopenicillin N was followed by ^{13}C n.m.r. and the extent of conversion was limited by the solubility of dioxygen in the solution. The tripeptide used in the latter procedure was labelled with ^{13}C in positions denoted by ● and ■ and also with ^{15}N (cf. Baldwin *et al.*, 1980).

(1)

(2)
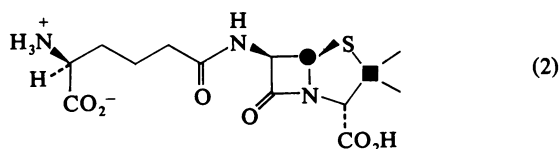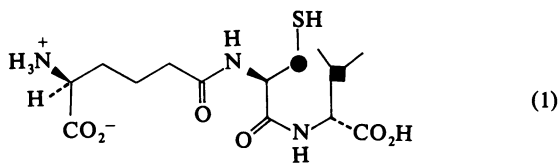
Materials and methods

A cell-free extract was obtained by disruption of *C. acremonium* C91 with glass beads in a Dyno-Mill at 5–10°C (Glen Creston, Stanmore, Middx., U.K.) and was partially purified (Abraham *et al.*, 1981). The resulting enzyme preparation was in 10 mM-

sodium 4-morpholinepropanesulphonate (Mops) buffer, pH 7.2.

The oxygen electrode used was from Rank Brothers, Bottisham, Cambridge, U.K. The electrode compartment containing the reaction mixture (2 ml maintained at 27°C) was a vessel with a magnetic stirrer and closed by a Perspex lid carrying a capillary opening for sampling. The reaction mixture contained the enzyme solution (1.82 ml, 0.48 mg of protein/ml), dithiothreitol (2.0 mM), L-ascorbic acid (1 mM) and δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (1 mM). The latter tripeptide was added last in 10 mM-acetic acid (100 μl). During the reaction, samples (50 or 100 μl) were taken in a syringe, diluted at once to 200 μl with 50 mM-sodium Mops (pH 7.2) and the reaction quenched by the addition of acetone (465 μl). After removal of the precipitated protein by centrifugation (200 g, 5 min) and concentration of the supernatant to 200 μl the sample was assayed for isopenicillin N against *Staphylococcus aureus* N.C.T.C. 6571 (Abraham *et al.*, 1981). A control experiment was carried out in which δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine was omitted from the incubation mixture.

δ-(L-α-Aminoadipyl)-L-[3-^{13}C]cysteinyl-D-[3-^{13}C, ^{15}N]valine (1) was synthesized as described by Baldwin *et al.* (1981). Its conversion into labelled isopenicillin N (2) was followed by monitoring the reaction in an n.m.r. spectrometer (Bruker WH 300) with a 10 mm tube. The parameters were: frequency, 75.47 MHz; sweep-width, 18.5 KHz; data length 16, 384 points; acquisition time, 0.4424 s; relaxation delay, 0.4 s; number of transients, 1000. The reaction mixture contained the enzyme preparation (3.7 mg of protein/ml), ^2H_2O (1.7 ml), dithiothreitol (2.0 mM), L-ascorbic acid (2.0 mM), $FeSO_4$ isotopically labelled δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (0.05 mM), (2.0 mM) and Mops buffer to a

total volume of 3.9 ml. The mixture was prepared at 0°C and then incubated in the spectrometer at 10°C.

Results and discussion

In one experiment with the oxygen electrode the initial concentration of the tripeptide (1) in the cell-free system (1 μmol/ml) was about four times the value given by Hickman (1978) for the concentration of O_2 (0.25 μmol/ml) in an air-saturated aqueous solution at 27°C. The assay values for isopenicillin N increased in parallel with the values for O_2 consumption during the first 20 min of incubation (Fig. 1) and corresponded to the syn-
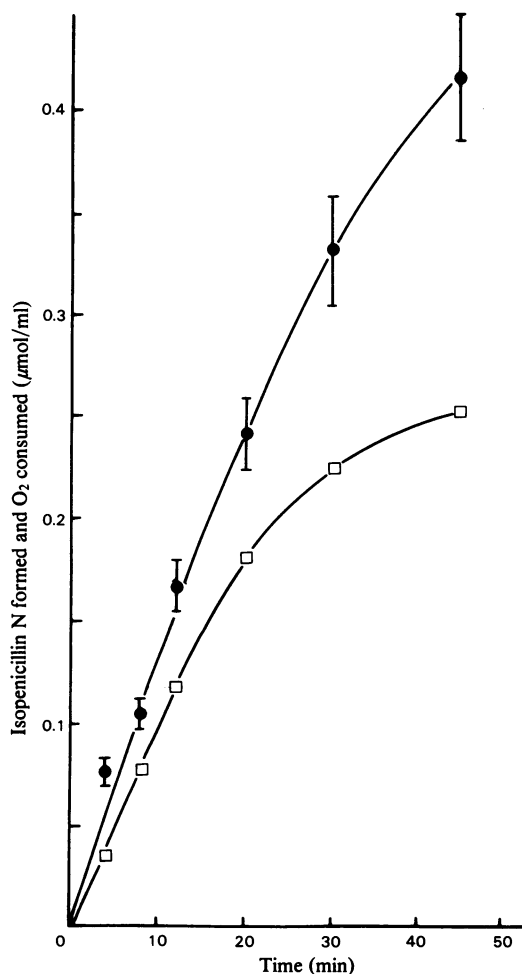
thesis of 1.33 mol of isopenicillin N/mol of dioxygen. However, the error in the assay of isopenicillin N is $\pm 15\%$ and the measured O_2 consumption could have been lower than the true value because of failure to exclude air during sampling and a tendency of air to leak into the incubation mixture as the O_2 content of the latter was progressively reduced by the reaction. This could also have been responsible for increases in the apparent value for (isopenicillin formed)/(O_2 consumed) to 1.40 and 1.48 after 25 min and 30 min respectively (Fig. 1).

A second experiment was carried out under the same conditions except that the amount of tripeptide (0.1 μmol/ml) was limited relative to O_2 (0.25 μmol/ml) and the experiment was terminated after 5 min incubation. The value obtained in this case for (isopenicillin N formed)/(O_2 consumed) was 1.14.

When the isotopically labelled tripeptide (2.1 μmol/ml) was incubated with the enzyme system in a 10 mm n.m.r. tube at 10°C the ^{13}C-signals due to the labelled peptide (1) at δ 26.5 and 31.4 p.p.m. decreased in intensity with time and those due to the labelled isopenicillin N produced at δ 65.1 and 67.3 p.p.m. increased with time (cf. Baldwin *et al.*,

Fig. 1. *O_2 consumption during the formation of isopenicillin from the tripeptide (1)*
O_2 consumption (□) at 27°C was measured with an oxygen electrode and isopenicillin N formation (●) by antibacterial assay. Before use in this experiment the enzyme preparation was subjected to gel filtration through Sephadex G-100 to remove a background consumption of O_2 by free ferrous iron.
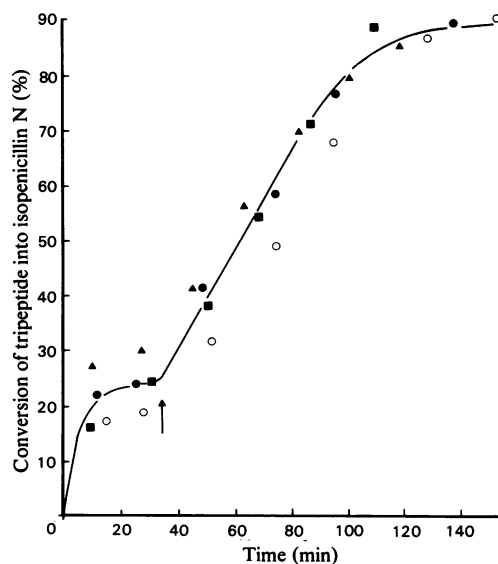

Fig. 2. *Conversion of the labelled tripeptide (1) into isopenicillin N (2)*
The conversion values represent the sum of the heights of the ^{13}C n.m.r. signals at 65.1 and 67.3 p.p.m. due to C-2 and C-5 of isopenicillin N expressed as a percentage of the combined signal height of reactant and product. The arrow indicates the initial aeration of the incubation mixture. ●, ▲, ■ and O represent the results of four different experiments at 10°C.

1980). After about 30 min the rate of conversion of the peptide into isopenicillin N levelled off, owing to the consumption of all the O_2 in the solution. Subsequent aeration of the mixture between each set of data accumulated resulted in the resumption of the conversion (Fig. 2). The levelling off of the rate of reaction due to lack of O_2 was observed when $23 \pm 5\%$ (average of four runs) of the peptide had been converted into isopenicillin N. This corresponds to $0.49 \pm 1 \mu$mol/ml of isopenicillin N. Since the initial air-saturated reaction mixture (prepared at $0°C$) contained 0.46μmol/ml of dissolved O_2 (Hickman, 1978) this result suggests that one molecule of dioxygen is consumed for every molecule of isopenicillin N produced.

The results obtained from these two separate determinations support the conclusion that one mol of dioxygen is used for the synthesis of one mol of penicillin, which is the stoichiometry expected from the loss of four hydrogen atoms from tripeptide (1) during its oxidative cyclization to isopenicillin N (2).

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