An Adenosine Triphosphate-Dependent Carbamoylphosphate–3-Hydroxymethylcephem *O*-Carbamoyltransferase from *Streptomyces clavuligerus*

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Cell-free supernatants from cells of *Streptomyces clavuligerus* (N.R.R.L. 3585), which are actively synthesizing cephamycin C, transfer a carbamoyl group from carbamoylphosphate to a 3-hydroxymethylceph-3-em-4-carboxylic acid nucleus to form a 3-carbamoyloxymethylcephem. This reaction was stimulated by nucleoside triphosphates and by a mixture of Mn^{2+} and Mg^{2+} cations. The enzyme responsible was purified 40-fold by batch absorption onto DEAE-cellulose and hydroxyapatite. The purified *O*-carbamoyltransferase is most active at pH 6.8. It is stabilized by phosphate anions, but is inhibited by PP_i anions, $(NH_4)_2SO_4$ or NaCl. The enzyme is stimulated by ATP, but it is not known whether this nucleotide acts as an effector or as a substrate. Some activity is observed with dATP, but two other analogues of ATP, in which a methylene group replaced the oxygen atom between the α - and β - or the β - and γ -phosphorus atoms, inhibit the action of ATP itself. The enzyme synthesizes a wide range of 3-carbamoyloxymethylcephems. The structure of some of these products, for example that of cefuroxime {3-carbamoyloxymethyl-7 β -[2-(fur-2-yl)-2-*syn*-methoxyiminoacetamido]ceph-3-em-4-carboxylic acid}, was confirmed by their proton-n.m.r. spectra.

A number of antibiotics, for example the mitomycins (Webb *et al.*, 1962), the polyoxins (Isono *et al.*, 1969) and cephamycin C (Nagarajan *et al.*, 1971; Miller *et al.*, 1972), contain an *O*-carbamoyl group. Although carbamoylphosphate and the *N*-carbamates such as citrulline are widely distributed, the esters of carbamic acid are unknown except as secondary metabolites of the Streptomycetes.

A range of semisynthetic 3-carbamoyloxymethylcephems (3-carbamoyloxymethylceph-3-em-4-carboxylic acids) have now been synthesized. One of these, cefuroxime, is very resistant to β -lactamases (O'Callaghan *et al.*, 1976; Neu & Fu, 1978) and exhibits a broad anti-bacterial spectrum under clinical conditions (Daikos *et al.*, 1977; Norrby *et al.*, 1977). These 3-carbamoyloxymethylcephems can be made by reacting a protected 3-hydroxymethylcephem with isocyanates such as trichloroacetylisocyanate (Brit. Patent, 1976). The conditions required for this reaction, and the need to remove the protecting group from the carbamate ester which is formed, prompted a detailed examination of the reaction catalysed by the Streptomycetes in their synthesis of cephamycin C. Our preliminary experiments showed that cell-free extracts of *Streptomyces clavuligerus* synthesize the ester from carb-

amoylphosphate (Brewer *et al.*, 1977a; U.S. Patent, 1978). We now show that the enzyme responsible is stimulated by ATP and is capable of carbamoylating a range of 3-hydroxymethylcephems.

Materials

[^{14}C]Carbamoylphosphate (8mCi/mmol) was obtained from New England Nuclear Inc., Boston, MA, U.S.A. Carbamoylphosphate and general laboratory reagents were obtained from BDH Chemicals, Poole, Dorset, U.K. Hydroxyapatite was prepared by the method of Atkinson *et al.* (1973). DE-23 DEAE-cellulose was obtained from Whatman, Maidstone, Kent, U.K. Weights of DEAE-cellulose were measured after vacuum filtration of aqueous slurries. Nucleoside triphosphates were from Sigma Chemical Co. Ltd., Kingston upon Thames, Surrey, U.K. and adenosine α,β -methylene triphosphate and adenosine β,γ -methylene triphosphate were from Miles Laboratories, Stoke Poges, Bucks., U.K. NE250 scintillation fluid was from Nuclear Enterprises, Sighthill, Edinburgh, U.K. Cellulose t.l.c. plates (no. 13254) were from Eastman Kodak, Kirkby, Liverpool, U.K. PM10 mem-

branes were from Amicon, High Wycombe, Bucks., U.K.

The cephalosporin substrates 7 β -[2-(fur-2-yl)-2-*syn*-methoxyiminoacetamido]-3-hydroxymethylceph-3-em-4-carboxylic acid (decarbamoylecfuroxime) (Cherry *et al.*, 1977), 3-hydroxymethyl-7 β -phenylacetamidoceph-3-em-4-carboxylic acid, 7 β -amino-3-hydroxymethylceph-3-em-4-carboxylic acid (see Turner *et al.*, 1978), 3-hydroxymethyl-7 β -(2-thienylacetamido)ceph-3-em-4-carboxylic acid, 7 β -[2-*syn*-carboxymethoxyimino-2-(fur-2-yl)acetamido]-3-hydroxymethylceph-3-em-4-carboxylic acid, 7 β -[2-*syn*-(1-carboxycyclobut-1-yloxyimino)-2-(fur-2-yl)acetamido]-3-hydroxymethylceph-3-em-4-carboxylic acid (Fig. 1) and the corresponding 3-carbamoyloxymethylcephems were obtained from Glaxo Group Research, Greenford, Middx., U.K.

Phosphate buffers

All phosphate buffers were prepared by mixing equimolar solutions of KH_2PO_4 and K_2HPO_4 .

Preparation of [3-hydroxymethyl- ^3H]deacetylcephalosporin C

Purified *Acremonium chrysogenum* deacetoxycephalosporin C dioxygenase (Turner *et al.*, 1978) (20 m-units) was incubated with [3-*Me*- ^3H]deacetoxycephalosporin C [7 β -(5-D-aminoadipamido)-3-methylceph-3-em-4-carboxylic acid] (1.25 μmol , 3.4 mCi), ascorbic acid (100 μmol), 2-oxoglutarate (100 μmol), FeCl_2 (1.0 μmol) and phosphate buffer (500 μmol) in a final volume of 11 ml.

After 17 h at 27°C acetic acid (2 ml) was added, the precipitated protein removed by centrifugation and the supernatant was evaporated to dryness. The product was dissolved in water (20 ml) and applied to a column containing 20 g of DEAE-cellulose equilibrated in 10 mM-phosphate buffer, pH 7.0. The column was eluted (20 ml/h) with a linear gradient (0–0.2 M, 200 ml) of NaCl in the same buffer. Fractions (6 ml) were collected and assayed with Dowex-1 acetate for [3-hydroxymethyl- ^3H]deacetylcephalosporin C [7 β -(5-D-aminoadipamido)-3-hydroxymethylceph-3-em-4-carboxylic acid] (Brewer *et al.*, 1977b). Peak fractions were pooled (18 ml), concentrated to 2 ml by rotary evaporation and applied to a Biogel P2 column (900 mm $\times$ 25 mm) and eluted with water (36 ml/h). Fractions (6 ml) were collected and assayed for product and peak fractions were pooled (60 ml) to yield 2.28 mCi of [3-hydroxymethyl- ^3H]deacetylcephalosporin C. Where necessary, this product was diluted with non-radioactive deacetylcephalosporin C.

Methods

Measurement of radioactivity

All samples were mixed with 15 ml of NE250, a dioxan-based scintillant. The radioactivity present in the sample was then measured in a Tri-Carb 3320 (Packard Instruments, Caversham, Berks., U.K.).

Protein determination

Protein was estimated by its absorbance at 280 nm.

Fig. 1. Cephalosporin substrates

The 7 β -side chains of all the other substrates are shown in Fig. 3 of Turner *et al.* (1978).

N.m.r. spectra

Spectra were recorded at 100 MHz with a Joel MH100 spectrometer. Samples were dissolved in [^2H]dimethyl sulphoxide and 3-trimethylsilylpropane-1-sulphonic acid (sodium salt) was used as the internal reference compound.

High-pressure liquid chromatography

Cephalosporins were analysed by high-pressure liquid chromatography and eluates were continuously monitored for radioactivity as described by Turner *et al.* (1978). The retention time for both 7 β -aminodeacetylcephalosporanic acid and decarbamoylcefuroxime was about 5 min, whereas that for the corresponding 3-carbamoyloxymethyl derivatives was about 6 min.

Assays of O-carbamoyltransferase with [3-hydroxymethyl- ^3H]deacetylcephalosporin C

Carbamoylphosphate and [3-hydroxymethyl- ^3H]deacetylcephalosporin C were incubated under various conditions with enzyme in 0.1 ml of water at 28°C. The reaction was stopped and unreacted deacetylcephalosporin C was lactonized for 30 min at 28°C with 0.1 ml of 1 M-HCl. The product was diluted with 4 ml of 10 mM-acetic acid and a sample (1 ml) was applied to a Dowex-1 acetate column (25 mm $\times$ 5 mm) which was eluted with 4 ml of 10 mM-acetic acid. Under these conditions the 3-carbamoyloxymethylcephem [7 β -(5-D-aminoadipamido)-3-carbamoyloxymethylceph-3-em-4-carboxylic acid] is retained on the column, whereas the lactone formed from the unreacted deacetylcephalosporin C is eluted. The radioactivity in 2 ml of the eluate was determined and the conversion of substrate into product was calculated from the difference between the radioactivity eluted from the incubated samples and that eluted from an unincubated control. Alternatively, the undiluted product (15 μl) was applied to a cellulose t.l.c. plate and eluted with propan-1-ol/acetic acid/water (5:1:2, by vol). The dried plate was cut into squares (10 mm $\times$ 10 mm), which were eluted with water (1 ml). The radioactivity present was then measured and the percentage conversion was calculated from the ratio of radioactivity in the spots with R_f values similar to authentic product and lactonized substrate.

Assay of O-carbamoyltransferase with [^{14}C]carbamoylphosphate

Decarbamoylcefuroxime (0.1 μmol), ATP (0.1 μmol), [^{14}C]carbamoylphosphate (0.1 μmol , 25 nCi), MgCl_2 (0.01 μmol) and MgSO_4 (0.01 μmol) and imidazole buffer adjusted to pH 6.75 with HCl (5 μmol) was incubated with enzyme in a final volume of 0.1 ml. The reaction was stopped after 15 min by the addition of 0.1 M-HCl (0.9 ml) and the [carbamoyl- ^{14}C]cefuroxime was extracted into ethyl

acetate (1.0 ml). A portion (0.5 ml) of the ethyl acetate layer was dried in a scintillation vial in a vacuum oven (40°C). Water (1 ml) was then added and the radioactivity present was determined. When corrected for substrate blanks, this radioactivity represents the incorporation of the [^{14}C]carbamoylphosphate into cefuroxime. Alternatively, the reaction mixture was treated with 1.0 M-HCl (0.1 ml) and incubated at 28°C for 30 min to lactonize the unreacted substrate. The cephalosporins were then extracted into ethyl acetate (1 ml), dried under vacuum, resuspended in ethyl acetate (40 μl) and a sample (20 μl) was analysed by high-pressure liquid chromatography. The ratio of the peaks at 260 nm due to cefuroxime and to the lactone formed from unreacted cephalosporin substrate was used to calculate the amount of carbamoylation that had occurred during the incubation.

Units of activity

The units of enzyme activity are calculated as μmol of product formed/min at 28°C.

Enzyme preparation

S. clavuligerus (N.R.R.L. 3585) shake-flask cultures were grown and harvested, and buffered cell-free supernatants were prepared as described by Brewer *et al.* (1977a).

Column-purified enzyme was prepared from 1.1 litre shake-flask cultures. Harvested cells (40 g) were washed in 10 mM-Tris buffer (adjusted to pH 7.2 with HCl), resuspended in 180 ml of the same buffer and disintegrated by stirring (2000 rev./min) for 1 min at 4°C in the 300 ml head of a Dynamill (Glen Creston, Stanmore, Middx., U.K.) with 255 ml of 0.1 mm glass beads. The broken-cell preparation was centrifuged at 4°C (30000 g for 30 min) and the supernatant (100 ml) was applied to a DEAE-cellulose column (200 mm $\times$ 50 mm) equilibrated with 10 mM-Tris buffer adjusted to pH 8.0 with HCl. The column was eluted (90 ml/h) with a linear gradient (0–1.0 M, 1.5 litre) of NaCl in the same buffer. Fractions (10 ml) were collected and assayed for *O*-carbamoyltransferase activity. Peak fractions were pooled (about 100 ml) and then concentrated to 10 ml by ultrafiltration on a PM10 membrane.

Batch-purified enzyme was obtained from 150 litre fermentations. *S. clavuligerus* was grown at 28°C on slopes containing 2.4% (w/v) Oxoid malt extract (Oxoid, London S.E.1, U.K.), 0.5% Oxoid yeast extract and 1.5% agar until spores were formed. The cells were then suspended in sterile water (10 ml) and a sample (2 ml) was transferred to a 250 ml baffled shake flask containing development medium (50 ml). The development medium, which contained 1% (w/v) glycerol, 0.5% Bibbys 3 $\times$ milled soya-bean meal (J. Bibby and Son, Liverpool 3, U.K.), 0.5% Oxoid yeast extract, 0.5% Oxoid tryp-

tone and 0.02% K_2HPO_4 in tap water, was adjusted to pH 6.5 with HCl before autoclaving. After growth in this medium for 48 h at 28°C while shaken on an orbital shaker (220 rev./min; 5 cm throw), samples (160 ml) of the culture were transferred to growth medium (4 litre) in 5 litre fermenters. The growth medium, which contained 3.5% (w/v) glycerol, 0.67% L-asparagine, 0.15% citric acid, 0.05% $MgSO_4 \cdot 7H_2O$, 0.042% KH_2PO_4 , 0.021% K_2HPO_4 , 0.02% $CaCl_2$, 0.01% NaCl, 0.01% $MnSO_4 \cdot 4H_2O$, 0.005% $ZnSO_4 \cdot 7H_2O$, 0.003% $FeSO_4 \cdot 7H_2O$ and 0.002% piperazine-1,4-diethanesulphonic acid, was adjusted to pH 7.0 with KOH before autoclaving for 30 min at 0.1 mPa (15 lb/in²). These cultures were stirred (250 rev./min with two 7.5 cm-diameter impellers) and aerated (360 litre/h) for 24 h at 28°C before a sample (6 litre) was transferred to fresh growth medium (150 litre). The culture was then aerated at 17 m³/h without stirring for 45 h at 28°C. The cells were harvested in a continuous-flow centrifuge (KA6; Westfalia Separator, Milton Keynes MK12 5PY, U.K.) and washed by passing 50 litre of 10 mM-imidazole adjusted to pH 7 with HCl through the centrifuge. The wet cell paste (about 5.5 kg) was suspended in the same buffer. The slurry (about 11 litre) was disintegrated by stirring (2000 rev./min) with 0.1 mm-diameter glass beads (500 ml) at 4°C in a Dynamill fitted with a 600 ml continuous-flow head (flow rate 100 ml/min). This yielded with washes, 12.7 litre of broken-cell preparation, which was treated with two successive batches (4 kg each) of DEAE-cellulose equilibrated with the imidazole buffer. The DEAE-cellulose was then washed twice with 8 litre of buffer and twice with 8 litre of buffer containing 0.1 M-NaCl. O-Carbamoyltransferase was eluted with three batches of 8 litre of 0.5 M-NaCl in buffer (Atkinson, 1973). To the 0.5 M-NaCl eluate was added 1 litre (settled bed volume) of hydroxyapatite suspended in 1 litre of

5 mM-phosphate buffer, pH 6.8. The supernatant (27 litre), which contained the O-carbamoyltransferase, was decanted off and precipitated with $(NH_4)_2SO_4$ at 55% saturation (9.6 kg), centrifuged (1000 g for 60 min at 4°C) and the precipitate (about 300 ml) was resuspended in 50 mM-phosphate buffer, pH 7.0, to yield 600 ml of enzyme preparation. After dialysis against the same buffer, the dialysis residue was centrifuged and the supernatant (570 ml) was retained.

Isolation of enzymically synthesized 3-carbamoyloxymethylcephems

7 β -Acylamino-3-hydroxymethylcephem (40 mg), ATP (disodium salt) (300 mg), carbamoylphosphate (dilithium salt) (150 mg), $MgSO_4 \cdot 7H_2O$ (20 mg), $MnCl_2 \cdot 4H_2O$ (20 mg) and imidazole (10 mmol) were adjusted to pH 6.75 with HCl and then incubated with batch-purified enzyme (2.1 units) in a final volume of 100 ml of water. After 4 h at 28°C the reaction was stopped by adding conc. HCl (2.5 ml). Any precipitate was centrifuged (3000 g for 10 min). The cephalosporins extracted from the supernatant with ethyl acetate (50–150 ml) were back-extracted into 0.1 M-NaHCO₃ (50 ml). The aqueous phase was washed with ethyl acetate (50 ml), acidified with 0.75 ml of conc. HCl and re-extracted into ethyl acetate. The organic extract was dried and the products obtained were recrystallized from methanol and washed thoroughly with diethyl ether.

Results

O-Carbamoyltransferase activity in cell-free supernatants from *S. clavuligerus*

Deacetylcephalosporin C will lactonize at acid pH and the lactone formed can be quantitatively separated from the 3-carbamoyloxymethylcephem by

Table 1. Measurement of O-carbamoyltransferase activity using [3-hydroxymethyl-³H]deacetylcephalosporin C [3-hydroxymethyl-³H]Deacetylcephalosporin C (100 pmol, 100 nCi) was incubated with carbamoylphosphate (3 μ mol), $MnCl_2$ (100 nmol) and cell-free supernatant from *S. clavuligerus* (40 μ l) in a final volume of 0.1 ml. The conversion of substrate into product was determined by using Dowex-1 acetate or t.l.c. as described in the Methods section.

O-Carbamoyltransferase assay					
Incubation time (min)	T.l.c.			Dowex-1 acetate	
	(c.p.m./peak)		Conversion (%)	c.p.m. in column eluate	Conversion (%)
	Lactonized substrate (R_F 0.4)	Product (R_F 0.2)			
0	7500	0	0	14 600	0
60	4030	3330	45.2	8140	44.2

chromatography either on a Dowex-1 acetate column (Brewer *et al.*, 1977b) or on cellulose t.l.c. plates. The *O*-carbamoyltransferase activity of a crude broken-cell supernatant was determined by these two methods (Table 1). The 44% decrease in [3-hydroxymethyl-³H]deacetylcephalosporin C lactone eluted from the Dowex columns corresponded well with the conversion into the 3-carbamoyloxy-methylcephem determined by t.l.c.

Effect of metal ions

The metal ion requirements of *O*-carbamoyltransferase were determined with a low concentration (0.25 μ M) of the high specific radioactivity (about 1 Ci/mmol) [3-hydroxymethyl-³H]deacetyl-

cephalosporin C. Mg²⁺ and Mn²⁺ stimulated the activity, the latter by as much as 5-fold (Table 2). On the other hand Zn²⁺, Cu²⁺ and Sn²⁺ (1 mM) inhibited the Mn²⁺-dependent activity by more than 60%, whereas Pb²⁺, Fe²⁺ and Ba²⁺ had little effect.

Effect of nucleoside triphosphates

O-Carbamoyltransferase activity could also be enhanced by the addition of some nucleoside triphosphates, ATP being the most effective (Table 2). When ATP, Mn²⁺ and Mg²⁺ were added together at a higher substrate concentration (10 μ M) the activity was increased about 20-fold (cf. Tables 1 and 3). Maximum activity was then obtained in the presence of both Mn²⁺ and Mg²⁺. With Mn²⁺ alone (or with 10 mM-Mg²⁺ alone) about 80% of the maximum activity was obtained, but with this less-sensitive assay (substrate at sp. radioactivity 25 mCi/mmol) no activity was detected unless both ATP and carbamoylphosphate were present (Table 3).

Enzyme purification

The stimulation of *O*-carbamoyltransferase activity by ATP, Mg²⁺ and Mn²⁺ allowed the introduction of a simple assay with [¹⁴C]carbamoylphosphate and decarbamoylcefuroxime substrates. When decarbamoylcefuroxime is incubated with [¹⁴C]carbamoylphosphate under these conditions, sufficient [carbamoyl-¹⁴C]cefuroxime is formed to allow its detection after extraction into ethyl acetate at acid pH. This contrasts with the similar assay using 7 α -methoxydeacetylcephalosporin C (Brewer *et al.*, 1977a), which required extensive purification of the radiolabelled product by high-pressure liquid chromatography. Assays based on [carbamoyl-¹⁴C]-cefuroxime extraction into ethyl acetate compared favourably with assays for cefuroxime based on high-pressure liquid chromatography (Fig. 2).

The cephalosporin substrate concentration (1 mM) in this new [¹⁴C]carbamoylphosphate assay was 100

Table 2. Effect of Mg²⁺, Mn²⁺ and nucleoside triphosphates on the *O*-carbamoyltransferase activity of the cell-free supernatant from *S. clavuligerus*

Cell-free supernatant from *S. clavuligerus* (50 μ l) was incubated in a final volume of 0.1 ml of water with [3-hydroxymethyl-³H]deacetylcephalosporin C (25 pmol), carbamoylphosphate (3 μ mol) and MnCl₂ (0.1 μ mol). The effects of additions and omissions on the *O*-carbamoyltransferase activity were determined by using Dowex-1 acetate as described in the Methods section. Results are calculated as ratios of the control activity obtained with no additions or omissions.

Assay conditions		<i>O</i> -Carbamoyltransferase activity (relative to control)
Additions (0.5 μ mol)	Omissions	
—	—	1.0
Mg ²⁺	Mn ²⁺	0.37
—	Mn ²⁺	0.21
ATP	—	3.0
UTP	—	2.6
CTP	—	2.2
GTP	—	1.0

Table 3. Effect of carbamoylphosphate, ATP, Mn²⁺ and Mg²⁺ on the *O*-carbamoyltransferase activity of cell-free supernatant from *S. clavuligerus*

Cell-free supernatant from *S. clavuligerus* (50 μ l) was incubated for 30 min in a final volume of 0.1 ml with [3-hydroxymethyl-³H]deacetylcephalosporin C (1 nmol, 25 nCi). Carbamoylphosphate (0.3 μ mol), ATP (0.1 μ mol), MnCl₂ (0.01 μ mol) and MgSO₄ (0.01 μ mol) were added as indicated and the amount of product formed was determined with Dowex-1 acetate.

Additions	<i>O</i> -Carbamoyltransferase activity (units/litre of enzyme)
Carbamoylphosphate	<0.03
Carbamoylphosphate, Mn ²⁺	<0.03
ATP, Mg ²⁺	<0.03
Carbamoylphosphate, ATP	0.19
Carbamoylphosphate, Mn ²⁺ , ATP	0.30
Carbamoylphosphate, Mn ²⁺ , ATP, Mg ²⁺	0.36

times greater than in the [3-hydroxymethyl-³H]deacetylcephalosporin C assay. This causes a 4-fold increase in the measured *O*-carbamoyltransferase activity. All activities therefore refer to the assay method described for each experiment.

Fig. 2. Measurement of *O*-carbamoyltransferase activity by using [¹⁴C]carbamoylphosphate

The activity of the column-purified *O*-carbamoyltransferase was determined by using [¹⁴C]carbamoylphosphate as described in the Methods section. The product, [carbamoyl-¹⁴C]cefuroxime, was estimated either from the radioactivity extracted into ethyl acetate (●) or from the *A*₂₆₀ peak areas obtained after high-pressure liquid chromatography of the cefuroxime and the lactonized substrate (○).

The *O*-carbamoyltransferase activity from a cell-free supernatant prepared from an *S. clavuligerus* shake-flask culture (1.1 litre) was purified by DEAE-cellulose column chromatography as described in the Methods section. The purification was followed by using the [3-hydroxymethyl-³H]deacetylcephalosporin assay. From a load containing 0.015 units (specific activity 5.5×10^{-6} units/(*A*₂₈₀), the enzyme (0.010 units) was eluted in a single peak at 0.25 M NaCl with a 12-fold increase in specific activity.

The results of a batch purification of *O*-carbamoyltransferase from a 150 litre culture of *S. clavuligerus* are summarized in Table 4. The variation in recovery throughout the purification is probably due to the inhibition by (NH₄)₂SO₄ (see below) and to the removal of contaminating phosphatases. By using this method a 98% yield of enzyme was obtained with a 40-fold increase in specific activity. For high enzyme yields it is essential that potassium phosphate be present during the hydroxyapatite adsorption step.

Stability of purified enzyme preparations

The purified *O*-carbamoyltransferase was unstable in the absence of phosphate anions. The column-purified enzyme, which was equilibrated with 10 mM-Tris buffer adjusted with HCl, lost all activity within 2–3 days at 4°C. When low concentrations of ATP and carbamoylphosphate (1 mM) were added to this enzyme full activity was retained for at least 3 days at 4°C. This was similar to the stability of the batch-purified enzyme which was equilibrated with about 1 mM-phosphate buffer and on which the low concentrations of ATP and carbamoylphosphate had little effect. However, if 50 mM-potassium phosphate pH 7.0 were added, the enzyme would remain fairly stable at 4°C and could be stored at -10°C for several weeks without significant loss of activity.

Table 4. Purification of *O*-carbamoyltransferase from *S. clavuligerus*

O-Carbamoyltransferase was purified from *S. clavuligerus* by the batch adsorption technique described in the Methods section. The enzyme activity was assayed with [¹⁴C]carbamoylphosphate. The low recovery of activity in the ammonium sulphate precipitate was probably the result of the inhibition of the *O*-carbamoyltransferase by ammonium sulphate (see Fig. 4).

	Volume (litre)	Protein concn. (<i>A</i> ₂₈₀)	O-Carbamoyltransferase activity		
			(units/ litre)	Recovery (units)	(units/ <i>A</i> ₂₈₀)
Broken-cell supernatant	12.7	112.2	1.98	25	1.8×10^{-5}
DEAE-cellulose eluate	24.0	10.3	0.74	18	7.2×10^{-5}
Hydroxyapatite supernatant	26.8	3.88	0.77	20	2.0×10^{-4}
(NH ₄) ₂ SO ₄ precipitate	0.57	61.2	26.0	15	4.3×10^{-4}
Non-diffusible material	0.60	57.6	41.4	24	7.2×10^{-4}

pH Optimum

A pH optimum of 6.75 was obtained for the purified *O*-carbamoyltransferase. About 75% of the full activity was observed at pH 5.9 and 7.4.

Nucleoside triphosphate specificity

Purified *O*-carbamoyltransferase was stimulated

Fig. 3. Effect of ATP on *O*-carbamoyltransferase activity. Batch-purified *O*-carbamoyltransferase (10^{-4} units) was assayed with either 0.1 μ mol (O) or 1 μ mol (●) of [14 C]carbamoylphosphate and with various amounts of ATP as indicated. The amount of product formed was estimated as described in the Methods section. The results are plotted as the percentage of the enzyme activity obtained with 1 mM-ATP.

by ATP additions up to a concentration of 1 mM. A further increase in the concentration of ATP then decreased the activity (Fig. 3). The optimum concentration was independent of the concentration of carbamoylphosphate.

In contrast with their effect on the crude cell-free supernatants, UTP and CTP were unable to activate the purified *O*-carbamoyltransferase when used at concentrations of 0.1 and 2 mM respectively. However, dATP could replace ATP to some extent, but no activity was detected in the presence of the methylene analogues of ATP. These analogues could also inhibit the enzyme activity in the presence of ATP, adenosine β,γ -methylene triphosphate being the most effective (Table 5).

Inorganic-ion inhibitors

The enzyme is inhibited by high salt concentrations (Fig. 4). $(\text{NH}_4)_2\text{SO}_4$ is especially effective. Under similar conditions, low concentrations of PP_i (5 mM) inhibit the enzyme by 85%, but the same concentration of phosphate has no effect.

Identification of product

A sample (15.3 mg) of cefuroxime was synthesized from decarbamoylcefuroxime (40 mg) and carbamoylphosphate as described in the Methods section. The n.m.r. spectrum of the product (Table 6) shows a downfield shift for the CH_2 group attached to $\text{C}_{(3)}$ of the cephem ring. This is characteristic of ester formation on the 3-hydroxymethyl group. The substrate itself shows no signal from the hydroxy proton of this 3-hydroxymethyl group due to its exchange with traces of water in [^2H]dimethyl sulphoxide. However, the product shows a new signal at 6.63 p.p.m. that is characteristic of the protons attached to the nitrogen atom of the carbamate. Finally, the splitting of the protons attached to $\text{C}_{(2)}$ of the cephem nucleus is a feature of the difference

Table 5. Effect of adenosine nucleoside triphosphates on purified *O*-carbamoyltransferase activity

DEAE-cellulose-column-purified *O*-carbamoyltransferase (5×10^{-6} units) was incubated for 1 h in a final volume of 0.1 ml with carbamoylphosphate (0.3 μ mol), MgCl_2 (0.01 μ mol), MnSO_4 (0.01 μ mol), imidazole (5 μ mol, adjusted to pH 6.75 with HCl) and [3-hydroxymethyl- ^3H]deacetoxycephalosporin C (1 nmol). Nucleoside triphosphates were also added as indicated and the amount of product formed was estimated by using Dowex-1 acetate (see the Methods section). The results are expressed as a percentage of the activity obtained with 1 mM-ATP.

Assay addition	Nucleotide concentration (mM) ...	O-Carbamoyltransferase activity (activity at 1 mM-ATP = 100)	
		0.1	1.0
ATP		44	100
dATP		10	10
α,β -Methylene-ATP		<5	<5
β,γ -Methylene-ATP		<5	<5
α,β -Methylene-ATP + ATP (1 mM)			62
β,γ -Methylene-ATP + ATP (1 mM)			39

between decarbamoylcefuroxime and cefuroxime itself, but it is not diagnostic for the presence of a carbamate ester.

Similar confirmation of the structures of 3-carbamoyloxymethyl-7 β -phenylacetamidoceph-3-em-4-carboxylic acid and 3-carbamoyloxymethyl-7 β -(2-thienylacetamido)ceph-3-em-4-carboxylic acid was also obtained after their synthesis in similar yield (15.2 and 22.2mg respectively) from the corresponding 3-hydroxymethylcephems.

The synthesis of the 3-carbamoyloxymethyl derivatives of 7 β -[2-*syn*-carboxymethoxyimino-2-

(fur-2-yl)-acetamido]-3-hydroxymethylceph-3-em-4-carboxylic acid and 7 β -[2-*syn*-(1-carboxycyclobut-1-yloxyimino)-2-(fur-2-yl)-acetamido]-3-hydroxymethylceph-3-em-4-carboxylic acid was detected by t.l.c. on silica eluted with chloroform/methanol/90% formic acid (90:16:4, v/v) but the products were not isolated.

The enzymatic synthesis of [carbamoyl- 14 C]7 β -amino-3-carbamoyloxymethylceph-3-em-4-carboxylic acid was confirmed by high-pressure liquid chromatography (Fig. 5). A new peak of radioactivity was detected. This absorbed at 260nm and had the same retention time as a standard sample of 7 β -amino-3-carbamoyloxymethylceph-3-em-4-carboxylic acid.

Discussion

Cells of *S. clavuligerus*, which are actively synthesizing cephamycin C, contain an *O*-carbamoyltransferase. This enzyme catalyses the synthesis of a 3-carbamoyloxymethylcephem from carbamoylphosphate and a 3-hydroxymethylcephem. In contrast with the dioxygenase, which oxidizes the 3-methyl group of deacetoxycephalosporin C to a 3-hydroxymethyl group (Turner *et al.*, 1978), the *O*-carbamoyltransferase is tolerant of structural alteration around the 7-amino group of the cephalosporin. Even 7-aminodeacetylcephalosporanic acid itself is a substrate, though some preliminary evidence suggests that those cephalosporins that carry 7-amido side chains with no net charge are the best substrates.

When purified the enzyme is fully active only in the presence of a mixture of Mn $^{2+}$ and Mg $^{2+}$ cations. It is also stimulated by ATP, but full activity is not observed when this nucleotide is present in excess (>1 mM). It can be replaced by dATP, but other

Fig. 4. Effect of $(\text{NH}_4)_2\text{SO}_4$ and NaCl on *O*-carbamoyltransferase activity

Batch-purified *O*-carbamoyltransferase (10^{-4} units) was assayed with [14 C]carbamoylphosphate essentially as described in the Methods section except that NaCl (●) or $(\text{NH}_4)_2\text{SO}_4$ (○) was added to the incubation mixture as indicated.

Table 6. Comparison of the ^1H n.m.r. spectrum of the substrate (decarbamoylcefuroxime) and the isolated product (cefuroxime) of the *O*-carbamoyltransferase

Abbreviation: dd, double doublet.

Decarbamoylcefuroxime				Cefuroxime			
δ (p.p.m.)	Intensity	Multiplicity	Assignment	δ (p.p.m.)	Intensity	Multiplicity	Assignment
3.63	2H	s	2-Methylene (cephem C $_{(2)}$)	3.50	1H	d, J18Hz	2-Methylene (cephem C $_{(2)}$)
3.95	3H	s	Methoxy (oxime)	3.70	1H	d, J18Hz	(cephem C $_{(2)}$)
4.31	2H	s	CH $_2$ OH (cephem)	3.94	3H	s	Methoxy (oxime)
5.20	1H	d, J5Hz	6-H (β -lactam)	4.66	1H	d, J12Hz	CH $_2$ OCONH $_2$ (cephem)
5.81	1H	dd, J8, 5Hz	7-H (β -lactam)	4.94	1H	d, J12Hz	
6.71	1H	dd, J3, 2Hz	Furyl protons	5.24	1H	d, J5Hz	6-H (β -lactam)
6.77	1H	d, J3Hz		5.85	1H	dd, J9, 5Hz	7-H (β -lactam)
7.89	1H	s		6.63	2H	s	OCONH $_2$ (carbamate)
9.82	1H	d, J8Hz	CONH (7-amido)	6.68	1H	dd, J3, 2Hz	Furyl protons
				6.76	1H	d, J3Hz	
				7.90	1H	s	
				9.82	1H	d, J9Hz	CONH (7-amido)

Fig. 5. High-pressure liquid chromatography of 7 β -amino-3-carbamoyloxymethylceph-3-em-4-carboxylic acid synthesized by *O*-carbamoyltransferase

7 β -Aminodeacetylcephalosporanic acid (40 μ g) was incubated in 0.1 ml of water with *O*-carbamoyltransferase (2×10^{-3} units), [14 C]carbamoylphosphate (dilithium salt) (150 μ g, 7.5 μ Ci), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (20 μ g), $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (20 μ g), ATP (disodium salt) (300 μ g) and imidazole (10 μ mol adjusted to pH 6.75 with HCl). After 4 h at 28°C the reaction was stopped with acetic acid (10 μ l) and the precipitate which formed was centrifuged. A sample of the supernatant (10 μ l) was analysed by high-pressure liquid chromatography as described in the Methods section. The absorbance (A_{260}) and radioactivity in the eluate were measured continuously after the injection of the sample (O). The main peaks in the eluate correspond to the solvent front (1), 7 β -aminodeacetylcephalosporanic acid (2) and 7 β -amino-3-carbamoyloxymethylceph-3-em-4-carboxylic acid (3). The delay between the detection of absorbance and of radioactivity (about 4 s) is overcompensated by the offset (2.5 mm = 7.5 s) between the pens on the double-pen recorder (see sample injection point). The radioactive trace therefore appears to be about 3 s in advance of the absorbance trace. Full scale deflection on the u.v. trace is equivalent to an A_{260} value of 0.5, and on the radioactivity trace is equivalent to 100 c.p.s. with a time constant of 3 s. The traces were copied directly from the original record to remove the chart grid-lines.

nucleotides such as GTP or UTP are only active in crude undialysed preparations where interconversion with ATP is possible. Those analogues of ATP in which methylene groups replace the oxygen atom between the α - and β - or the β - and γ -phosphorus atoms inhibit the action of ATP itself.

The role of ATP in the reaction is not clear. This nucleotide is not a substrate for either the aspartate or the ornithine *N*-carbamoyltransferase, although it does activate the former enzyme (see Wiame *et al.*, 1973). The preparations from *S. clavuligerus* degrade ATP to ADP and AMP during the synthesis of the 3-carbamoyloxymethylcephems. However, all of this degradation may be due to the presence of contaminating enzymes such as phosphatases and carbamoylkinase. It is therefore not unlikely that the ATP is merely acting as an effector.

Carbamoylphosphate now seems the most likely precursor of the carbamate ester in two other antibiotics, mitomycin and polyoxin. When whole cells of *Streptomyces verticillatus* are actively synthesizing mitomycin they transfer the carbamoyl group of citrulline into this antibiotic as a single unit with little change in specific activity (Hornemann & Eggert, 1975). The reversal of ornithine transcarbamoylase would yield the carbamoylphosphate, which was postulated as an intermediate, but other pathways, such as the direct transfer of the carbamoyl group from citrulline, were not excluded. This same route would also explain why crude homogenates of *S. clavuligerus* can synthesize cephamycin C from 7 β -aminoadipamido-3-hydroxymethyl-7 α -methoxyceph-3-em-4-carboxylic acid and citrulline. By comparison the direct esterification with carbamoylphosphate is more efficient (Brewer *et al.*, 1977a; U.S. Patent, 1978), and is the one which is important in the synthesis of polyoxin by *Streptomyces cacaoi*. When cell-free extracts of *Streptomyces cacaoi* are treated with $(\text{NH}_4)_2\text{SO}_4$ the precipitate will incorporate [14 C]carbamoylphosphate directly into 2-amino-5-carbamoyloxyvaleric acid, a precursor of the polyoxins (Funayama & Isono, 1977). The enzyme that catalysed this reaction was clearly not ornithine *N*-carbamoyltransferase, but it was unstable and it could not be purified.

The *O*-carbamoyltransferase from *S. clavuligerus* allows the direct synthesis of an unprotected carbamoyloxymethylcephem. For its most efficient use on a large scale the enzyme would need to be immobilized and the consumed reagents replaced. Carbamoylphosphate could be synthesized from KH_2PO_4 and KCNO (Spector *et al.*, 1957), but if the regeneration of ATP were necessary this would present a more difficult, though not an insuperable, challenge (Pace *et al.*, 1976; Butler, 1977). The practical advantage of the enzymic process is obvious, and it is the subject of a patent application (U.S. Patent, 1978).

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