Biotransformation, by *Enterobacter agglomerans*, of pyrimidine acyclonucleosides to acyclonucleotides

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Abstract. The ability of *Enterobacter agglomerans* to transform naturally occurring nucleosides into nucleotides has been utilized to transform newly synthesized pyrimidine acyclonucleosides into the corresponding acyclonucleotides. Unselected bacteria were used as the source of nucleoside phosphotransferase, the phosphate group donor being 4-nitrophenyl phosphate in the presence of Zn^{2+} ions. Optimal reaction conditions are outlined.
Key words. Nucleoside phosphotransferase; enzymic synthesis of acyclonucleoside phosphates.

Acyclovir (ACV, acycloguanosine)[1], an acyclonucleoside that is effective against herpesvirus, is converted to biologically active acyclonucleotides that inhibit viral DNA polymerase in virus-infected human cells[2,3]. The search for new antiviral drugs has led to the synthesis of a series of acyclonucleosides[4–8], not all of which, however, are converted in vivo to acyclonucleotides[9]. They have therefore been phosphorylated chemically or biochemically in vitro and the acyclonucleotides thus obtained have been tested for antiviral activity[6–8]. Novel pyrimidine acyclonucleosides synthesized in our institute have been phosphorylated[10], using the chemical method of Yoshikawa et al.[11], to obtain standard acyclonucleotides. For purposes of comparison, an attempt has also been made to convert the initial acyclonucleosides to acyclonucleotides by biotransformation using bacteria.

Many bacteria are able to biotransform natural nucleosides into nucleotides; some of these bacteria, e.g. *Bacillus subtilis* and *Brevibacterium ammoniagenes*, are already employed in biotechnological processes[12]. The bacterium that has been chosen for the procedures described here – *Enterobacter agglomerans* (formerly *Erwinia herbicola*) – has not previously been used in biotechnology; it is readily available, easy to culture and provides a rapid and efficient supply of nucleotides[13–15]. No selection of the most active strains has been attempted because the main purpose of the investigation was to determine the effectiveness of the species as a whole, as a source of acyclonucleotides.

Materials and methods

Eight typical pyrimidine acyclonucleosides with structures representative of the group as a whole (fig. 1) were selected for investigation from a number synthesized in our institute[7]. Cells of *E. agglomerans* from our culture collection were grown on agar slopes under standard conditions and suspended in 0.85% NaCl (1×10^9 cells/ml). All glass vessels, solutions and substrates were subjected to routine sterilization.

Biomass for use in the investigations was obtained from three-dimensional cultures on the medium described by Popov et al.[14] (beef-peptone broth amended with 0.5% yeast extract) at pH 7.6–7.8. The basic medium was inoculated with the (experimentally matched; see later) cell suspension. Cultures were grown in 100-ml conical flasks and 25 ml volumes of base material for 18 h at 26 °C, using a thermostatic shaker (180 rpm). The biomass was spun down (2500 × g for 10 min), washed in 0.9% NaCl and resuspended in all small volume of the solution. The collected suspensions were centrifuged as described above and stored at – 20 °C.

Mixtures investigated comprised acyclonucleoside (3.8 µmol), Na_2-4-nitrophenylphosphate (Koch-Light Lab. Ltd. UK; 46 µmol), biomass (19 mg dry weight), 1 M $ZnSO_4$ (10 µl) and 0.2 M acetate buffer (to a total volume of 1 ml), pH 4.5. Mixtures were incubated for 4.5 h at 37 °C, after which the biomass was spun down, as described above. The supernatant was cooled in ice-cold water, deproteinized with 0.1 volume of 30%

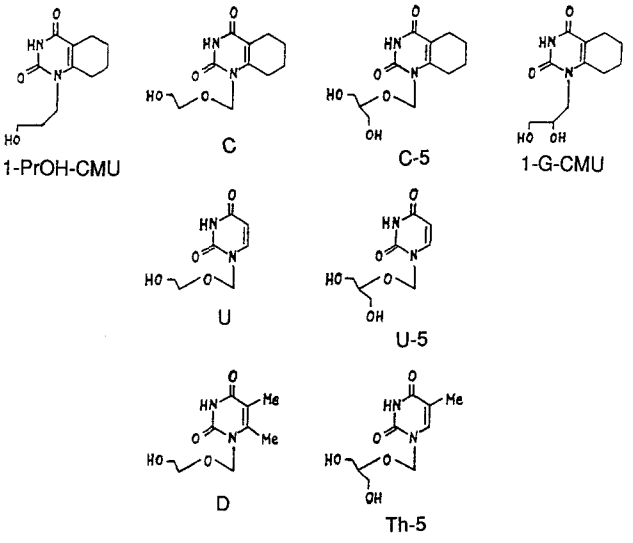

Figure 1. Structures of the acyclonucleosides investigated. Codes employed: 1-PrOH-CMU: 1-N-(3′-Hydroxypropyl)-5,6-tetramethyleneuracil, C: 1-N-[(2′-Hydroxy)ethoxymethyl]-5,6-tetramethyleneuracil, C-5: 1-N-[(1′,3′-Dihydroxy)-2′-propoxymethyl]-5,6-tetramethyleneuracil, 1-G-CMU: 1-N-(2′,3′-Dihydroxypropyl)-5,6-tetramethyleneuracil, U: 1-N-[(2′-Hydroxy)ethoxymethyl]uracil, U-5: 1-N-[(1′,3′-Dihydroxy)-2′-propoxymethyl]uracil, D: 1-N-[(2′-Hydroxy)ethoxmethyl]-5,6-dimethyluracil, Th-5: 1-N-[(1′,3′-Dihydroxy)-2′-propoxymethyl]-5-methyluracil.

Yields and R_f values of acyclonucleotides obtained with *E. agglomerans* biotransformation

Codes of initial compounds +	Yields of obtained acyclonucleotides* (mol%)	R_f values** on TLC plates: Silica gel (1) I	Silica gel (1) II	Cellulose (1) I	Cellulose (1) II	Cellulose (2) I	Cellulose (2) II
1-PrOH-CMU	46.1(16.7)	0.79	0.37	0.96	0.42	–	–
C	47.3(59.1)	0.75	0.36	0.96	0.40	–	–
C-5	44.3(23.0)	0.69	0.35	0.93	0.44	0.72	0.18
1-G-CMU	41.5(22.2)	0.69	0.40	0.90	0.45	0.64	0.19
U	41.4(40.0)	0.55	0.24	0.74	0.30	–	–
U-5	37.5(19.6)	0.56	0.24	0.72	0.29	0.38	0.05
D	42.5(65.4)	0.71	0.33	0.90	0.41	–	–
Th-5	55.1(21.6)	0.60	0.32	0.80	0.36	0.65	0.12
4-Nitrophenylphosphate	– –	–	0.60	–	0.75	–	–
4-Nitrophenol	– –	0.84	–	0.95	–	–	–
Inorganic phosphate	– –	–	0.05	–	0.15	–	–

* Yields of the same products obtained during chemical phosphorylation[10] are shown in parentheses. Biotransformations of dihydroxyl acyclonucleosides give only monophosphates (racemic mixtures); however, in chemical phosphorylations, bis- and cyclic phosphates were also obtained as side products.

** The Roman numeral I refers to the initial compounds and II to the corresponding acyclonucleotides. The Arabic numerals 1 and 2 refer to the solvent systems described in the text[16]: 1, main (alkaline) solvent system; 2, acidic solvent system.

+ See figure 1.

$HClO_4$, adjusted to a pH of about 7.0 with 15% KOH and recentrifuged, as before. The resultant clear supernatant was then applied to thin-layer chromatography (TLC) silica gel plates (Art 5715, E. Merck, Germany). A preliminary developing system was used to eliminate the 4-nitrophenol that was produced during the reaction and the acyclonucleotide products were isolated by the main developing system[16]. Spots corresponding to those from standard compounds obtained by chemical phosphorylation were detected under UV light (254 nm)[10] and subsequently scraped off the plate and eluted with distilled water. The absorbance of the eluate was measured and the product yield calculated (see table). The eluates were then concentrated under reduced pressure and analysed by TLC on silica gel and cellulose plates (Art 5728) in the main developing system[16]. The R_f values obtained are shown in the table. Eluates containing phosphorylation products of dihydroxyl acyclonucleosides were subjected to two-dimensional TLC on cellulose plates, using an alkaline 'main' solvent system[16] and an acidic secondary solvent system[16]. Product spots were authenticated by treatment with a molybdate reagent[17] (which also develops the spots of inorganic phosphates). The products obtained were chromatographically homogeneous and identical to those from corresponding products obtained by chemical phosphorylation.

Results and discussion

The cells of *E. agglomerans* contains nucleoside phosphotransferase (EC 2.7.1.77) as a bound component of the cell wall[13], which transports the PO_4^{3-} group from a corresponding donor substance, generally 4-nitrophenyl phosphate (NPP) to first-order hydroxyl nucleoside groups. It was to be expected, therefore, that our compounds, being proximate nucleoside analogues, would also be substrates for phosphotransferase.

In order to culture *E. agglomerans* it was necessary to devise inoculation methods that had not been described by previous authors[13–15]. To this end, five flasks were inoculated with the cell suspension (0.05, 0.1, 0.15, 0.2 and 0.25 ml) at a cell density of 10^9 cells/ml. After culture at 28 °C had been terminated, as previously described[13–15], the biomass was centrifuged at 2500 × g and weighed (the previous authors[13–15] had used 4500 × g, which we considered to be excessive). The experiment was repeated three times. The greatest yields of biomass (mean 163 mg) were obtained from flasks inoculated with 0.15 ml suspension.

To find the relevant conversion factor, 10 biomass samples were weighed, then dried at 37 °C for 4 h and reweighed. The mean $mass_{moist}/mass_{dry}$ ratio was close to 9, which factor was subsequently utilized when reaction mixtures were assembled (calculations have not been given by the previous authors[13–15]). With the incubation temperature reduced to 26 °C, as in *Mycota* cultures, there was extensive (50%) growth of biomass. The experiment was repeated 10 times (after inoculation with 0.15 ml suspension) and ca 230–250 mg biomass was obtained from each flask. A tentative U-compound biotransformation was carried out to ascertain whether the change in incubation temperature had affected the cell phosphotransferase activity. Biomass was grown at 26 and 28 °C. (U compounds were chosen because of their simple syntone structure and unmodified aglycone component.)

We used the experimental conditions described by Popov et al.[14], which, in fact, involved long transformation times because of the lack of Zn^{2+} ions; however, the conditions make allowance for various incubation temperatures, including the 37 °C used in our study. Other authors[15] obtained more rapid transformation in the presence of Zn^{2+} ions, but this was at 50 °C, which we considered to be unsafe in view of the general instability of our compounds. Our test mixture comprised U compound (38 µmol), NPP (220 µmol), biomass (42 mg on a dry-weight basis), and 0.2 M acetate buffer, pH 4.5, to a

total of 1 ml. Incubation was for 16 h at 37 °C. In both cases, an identical yield (32.3 %) of U-compound phosphate was obtained, which proved that the two biomass types, cultured at 26 °C and 28 °C, had identical phosphotransferase contents and also gave preliminary confirmation of the suitability of *E. agglomerans* for acyclonucleoside biotransformation. The acyclonucleosides chosen for the present study were subjected to biotransformation using a rapid procedure described by Kvasyuk[15]. Before this could be done, however, it was necessary to determine the optimum reaction time at the conjectural temperature of 37 °C. The weight of biomass used had also to be optimized to ensure biotransformation of 1 µmol of substrate. Previous authors[13–15] had used amounts varying (on a dry-weight basis) from 0.67 to 2.63 mg/µmol for the same substrate.

For a number of acyclonucleosides, i.e. U, C-5, Th-5 (fig. 1), and nucleosides dThd, Urd, five mixtures were prepared, with components according to previous recommendations[15]: acyclonucleoside or nucleoside, 3.8 µmol; NPP, 46 µmol; biomass (dry weight) 10 mg; $ZnSO_4$, 10 µmol, and 0.2 M acetate buffer (pH 4.5) to 1 ml. Mixtures were incubated at 37 °C and the progress of the reaction was monitored hourly using alkaline method chromatography on silica gel plates in conjunction with UV spectrophotometry. The results are depicted in figure 2 a. Five analogous mixtures were subsequently assembled, with U-compound (3.8 µmol) as the test substance and, successively, 3.8, 7.6, 11.4, 15.2 and 19 mg (dry weight basis) of biomass. The mixtures were incubated at 37 °C for the predetermined optimum time (4.5 h). As before, the effect on the amount of U-compound phosphate obtained, of the relationship between dry weight of biomass per µmol acyclonucleoside, was investigated. The results shown in figure 2 b show the linearity of the relationship. An optimum value of 5 mg 'dry' biomass/µmol acyclonucleoside was accepted. Volumes of biomass greater than this are useless because the post-reaction mixture forms a dense pulp which is difficult to process.

During the study it was found that separation of post-reaction mixtures on TLC plates is markedly disturbed by the formation of a protein film; for this reason, a deproteinization procedure (which was not employed by the original authors) using 30 % $HClO_4$ was introduced.

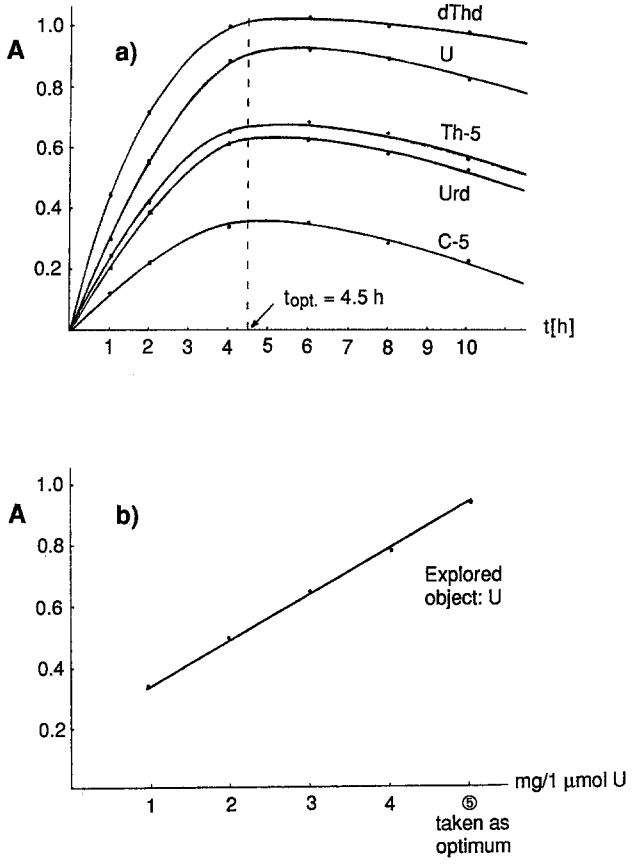

Figure 2. Optimisation of *E. agglomerans* parameters for biotransformation of several acyclonucleosides and nucleosides using 4-nitrophenylphosphate as the PO_4^{3-} donor in the presence of Zn^{2+} (incubation conditions as in the text), where A is the absorbance of product spot eluates from TLC chromatograms at its λ_{max}: a) incubation time, b) weight relation of dry biomass per 1 µmol acyclonucleoside or nucleoside (where m_B is the weight quantity of biomass, therefore number 1 marks 1 mg of dry biomass per 1 µmol U, number 2–2 mg per 1 µmol U and so on).

Conclusions

The traditional biochemical methods of acyclonucleotide production are extremely time- and labour-consuming. Among other problems, a difficult isolation procedure for the enzyme is necessary, together with additional determinations (such as protein content and enzyme activity) in order to obtain reproducible results. The method described here is free from such drawbacks. The role of the enzyme preparation is taken on here by an undisturbed biological mass of bacteria grown for 24 h, which take no more than 5 h to prepare for this purpose. The cells are derived from cultures in the exponential growth phase and thus are of constant protein composition, thereby obviating the above-mentioned additional determinations.

The reaction mixtures have short incubation times, and deproteinization enables their products to be separated on chromatographic plates or chromatography paper. The product yields are generally higher than those yields obtained from chemical phosphorylation.

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Suppression of chaos by periodic oscillations in a model for cyclic AMP signalling in *Dictyostelium* cells

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Abstract. We investigate how the introduction of cells oscillating periodically affects the behaviour of a suspension of *Dictyostelium discoideum* amoebae undergoing chaotic oscillations of cyclic AMP. The analysis of a model indicates that a tiny proportion of periodic cells suffices to transform chaos into periodic oscillations in such suspensions. A similar result is obtained by forcing the aperiodic oscillations by a small-amplitude, periodic input of cyclic AMP. The results provide an explanation for the observation of regular oscillations in suspensions of a putatively chaotic mutant of *Dictyostelium discoideum*[12]. More generally, the results show how chaos in biological systems may disappear through the coupling with periodic oscillations.
Key words. Dictyostelium discoideum; cAMP oscillations; biological rhythms; coupled oscillators; chaos; nonlinear dynamics.

In contrast to wild type *Dictyostelium discoideum* amoebae which display periodic waves of chemotactic movement in response to pulses of cyclic AMP (cAMP) emitted at regular intervals by aggregation centers after starvation[1–4], the mutant *Fr 17* aggregates in an aperiodic manner[5]. Preliminary studies of suspensions of the related mutant *HH 201* provided evidence for erratic oscillations of cAMP[6]. The occurrence of chaotic oscillations in a model for cAMP signalling in *D. discoideum* suggested that the aperiodic behaviour of the mutants *Fr 17* and *HH 201* might provide an example of autonomous chaos at the cellular level[7,8] (in most examples known in biology[9–11], chaos is nonautonomous as it results from the periodic forcing of an oscillatory system). The possible occurrence of chaos in *Dictyostelium* was tested by studying light scattering changes, which accompany cAMP oscillations, in suspensions of the mutant *HH 201*[12]. Regular oscillations similar to those occurring in the wild type were observed in these experiments instead of chaos. By analyzing the model for cAMP oscillations we show that these observations might be explained by the fact that a tiny proportion of periodic cells suffices to suppress chaos in cell suspensions. This result is of general significance as it shows how chaos in chemical or biological systems can disappear through the coupling with periodic oscillations.

The model for cAMP oscillations in *Dictyostelium*[13] is based on the positive feedback exerted by extracellular cAMP on its intracellular production[14], and on desensitization of the cAMP receptor through reversible phosphorylation[15] (for a scheme of the model, see also fig. 1 in Martiel and Goldbeter[7]). In addition to accounting for periodic oscillations of cAMP and for the relay of suprathreshold cAMP pulses[13,16], this model predicts the occurrence of more complex phenomena such as the coexistence of two stable rhythms[17], and complex oscillations in the form of bursting or chaos[7,8]. The model has also been used to simulate propagating waves of cAMP in the course of slime mould aggregation on agar[18,19].

To examine the effect of the presence of periodic cells in a suspension of chaotic amoebae, we consider the simplest case of mixing two populations, one periodic and the other chaotic (the results generalize to the mixing of a larger number of distinct populations); the situation considered is schematized in figure 1. The two populations differ by the value of a single parameter, which is either the net rate of ATP supply within the cells (v), or