# ISOLATION OF L-LACTYL-PUROMYCIN FROM *BACILLUS SUBTILIS* AND YEAST

G.KREIL, H.BACHMAYER, K.A.DAVIS and G.POLZ
*Institute of Molecular Biology, Austrian Academy of Sciences, Wasagasse 9, 1090 Vienna, Austria*

Received 17 June 1968

## 1. Introduction

We have recently observed the formation of N-formyl-methionyl-puromycin in intact bacterial cells incubated with puromycin and radioactive methionine [1]. According to present evidence this represents a simple test for chain initiators *in vivo*. Following a preliminary report implying that N-acetyl-alanine could act as chain initiator in *Bacillus subtilis* [2], we tried to substantiate this with our approach. Intact cells of *B. subtilis* were incubated with puromycin and $^{14}$C-alanine. Clearly, a labelled product was formed, the yield of which was dependent on the concentration of puromycin in the incubation medium. After total hydrolysis of this compound, however, all the label was recovered as lactic acid.

The present communication reports the formation of L-lactyl-puromycin by intact cells of *B. subtilis* and baker's yeast incubated with puromycin and $^{14}$C-(D,L)-lactate. Some possible implications of this finding are briefly discussed.

## 2. Materials and methods

Materials used: Uniformly labelled $^{14}$C-(L)-alanine and 1-$^{14}$C-(D,L)-lactate (The Radiochemical Centre), methoxy-$^{3}$H-puromycin (New England Nucl. Corp.), puromycin-di-hydrochloride (Nutritional Biochemicals Corp.), Pronase (B grade, Calbiochem), carboxypeptidase A (2 × cryst., Worthington), Nagarse (Nagarse Co.), Glusulase (Endo Labs.), rabbit muscle lactate dehydrogenase, yeast alcohol dehydrogenase and pig heart pyruvic-glutamic transaminase (Boehringer). Prior to use, $^{3}$H-puromycin was purified by paper electrophoresis at pH 6.5 (10 mM Na-phosphate buffer). $^{14}$C-(D,L)-lactyl-$^{3}$H-puromycin was obtained by the following procedure: 0.4 μmoles of puromycin-di-HCl containing approx. 10 μC $^{3}$H-puromycin and 0.04 μmoles $^{14}$C-lactate (approx. 1 μC) were dissolved in 0.1 ml of 0.5 M triethylamine, taken to dryness and redissolved at −5° in 0.1 ml of abs. dimethylformamide containing 0.1 μmole dicyclohexylcarbodiimide. The solution was kept in an ice bath and subsequently at room temperature, each for 30 min. Lactyl-puromycin was separated from excess puromycin by paper electrophoresis at pH 1.8. Using the same conditions, $^{14}$C-lactyl-glycyl-puromycin was prepared from glycyl-puromycin and $^{14}$C-lactate.

*B. subtilis* (ATCC 6051) was grown to late log phase in nutrient broth with 0.25% glucose. Cells were harvested, washed and incubated for 30 min at 37° in 2% glucose containing 5 to 10 μC radioactive alanine or lactate and various amounts of puromycin neutralized with KOH. Per sample, 20 to 40 mg of wet cells were used. *Saccharomyces cerevisiae* (wild type strain W) was cultured to late log phase in a half synthetic medium [3], containing 0.3% yeast extract (Difco) and 0.8% glucose. Yeast protoplasts were obtained by incubating 200 mg of wet cells with 10 ml of 1.6 M sorbitol (pH 5.8), containing 1% Glusulase, at 30° for 15 min. Protoplasts were washed twice with sorbitol, divided into four aliquots and incubated for 40 min at 30° in 0.5 ml sorbitol containing puromycin and radioactive lactate.

With both organisms the incubated cells were subsequently diluted with 1 ml of 0.1 M Na-acetate

buffer (pH 5.5) and extracted three times with 1 volume of ethyl acetate. The extract was dried and fractionated by paper electrophoresis at pH 1.8. Under these conditions, lactyl-puromycin migrates to the cathode, just short of DNP-arginine, which was used as visible marker. The paper was cut into sections, 1.5 to 2 cm wide, and the radioactivity of each section determined in a liquid scintillation counter. Peak fractions were eluted and investigated further by paper electrophoresis at pH 3.5 and 4.8 in pyridine/acetic acid buffers and by paper chromatography in *n*-butanol/acetic acid/water (4/1/5). Digestions with Pronase, Nagarse and carboxypeptidase were run with 0.2 mg of the respective enzyme in 0.5 ml 5 mM Tris buffer at pH 8.0 and 37° for various periods of time.

Partial conversion of the lactate, isolated after Pronase digestion, to alanine was accomplished in 1 ml Tris buffer (5 mM, pH 8.5) containing 10 μg L-lactate dehydrogenase, 15 μg alcohol dehydrogenase, 7 μg pyruvic-glutamic transaminase, 5 μmoles acetaldehyde, 0.5 μmoles NAD and 2.5 μmoles glutamic acid. This reaction mixture was incubated at 30° for 20 min, dried and fractionated by paper electrophoresis at pH 1.8.

## 3. Results

Intact cells of *B. subtilis* were incubated in 2% glucose in the presence of various amounts of puromycin (1 to 20 mM) and radioactive L-alanine or D,L-lactate. The ethyl acetate extract was fractionated by paper electrophoresis at pH 1.8. Only one radioactive peak could be observed, which comigrated exactly with synthetic lactyl-puromycin. The amount of label recovered in this fraction was dependent on the concentration of puromycin used. In a typical experiment with $^{14}$C-lactate, the yield was 50, 1420, 2750 and 7510 counts/min at puromycin concentrations of 0, 1, 4 and 20 mM, respectively. Up to 0.1% of the total label added was incorporated into this compound. For further characterization, the material migrating with synthetic lactyl-puromycin was eluted and fractionated by paper electrophoresis at pH 3.5 and 4.8 and by paper chromatography. Under all these conditions the radioactive compound isolated from *B. subtilis* was indistinguishable from synthetic lactyl-puromycin. In double label experiments, where $^{3}$H-puromycin was used in conjunction with $^{14}$C-lactate, it could be shown that both types of label were incorporated into this compound. After total hydrolysis in 6 N HCl at 105° for 4 hours, or after digestion with Pronase for 15 hours, over 90% of the $^{14}$C-label could be recovered as lactic acid by paper electrophoresis at pH 3.5 and 4.8 and by paper chromatography. Moreover, in a system containing L-lactate dehydrogenase, NAD, alcohol dehydrogenase plus acetaldehyde (to trap the NADH), glutamic acid and pyruvic-glutamic transaminase, the radioactive substance isolated from the Pronase digest was partly converted to alanine. In one experiment, 50.4% of the radioactivity was recovered as alanine after incubation in the above system. Under the same conditions, using authentic, radioactive D,L-lactate, 51.6% of the L-isomer was converted to alanine. In a control, without lactate dehydrogenase less than 1% of the material isolated from the Pronase digest was in the alanine fraction.

Digestion of the puromycin derivative from *B. subtilis* with Nagarse for 15 hr yielded a small amount (6 to 10% yield) of a new compound which was detected half-way between free lactic acid and the origin upon paper electrophoresis at pH 4.8. In double label experiments, both $^{14}$C- and $^{3}$H-counts were present in this compound and it is therefore considered to be N-lactyl-O-methyl-tyrosine. This substance could be further digested with carboxypeptidase A to yield $^{14}$C-lactate and $^{3}$H-O-methyl-tyrosine. All experiments with proteolytic enzymes could be duplicated starting with synthetic $^{14}$C-(D,L)-lactyl-$^{3}$H-puromycin.

In order to exclude the possibility that we are actually isolating a lactyl-aminoacyl-puromycin, the simplest compound of this type, $^{14}$C-lactyl-glycyl-puromycin, was synthesized. Paper electrophoresis at pH 1.8 separates lactyl-puromycin from the somewhat slower moving lactyl-glycyl-puromycin. Other lactyl-aminoacyl-puromycins containing a neutral amino acid with greater molecular weight than glycine can be expected to migrate even slower under these conditions. Lactyl-aminoacyl-puromycins containing acidic or basic amino acids would separate from lactyl-puromycin upon electrophoresis at pH 3.5 or 4.8.

In similar experiments with yeast protoplasts, the same compound could be observed which was by all the criteria described above indistinguishable from L-lactyl-puromycin. However, in the case of yeast, this compound is only formed from 1-$^{14}$C-(D,L)-lactate. We were so far unable to observe any incorporation of label from $^{14}$C-alanine. Lactyl-puromycin could also be isolated from intact yeast, incubated with puromycin and labelled lactate, but yields were about half those observed with protoplasts at any given concentration of puromycin.

As controls, $^{14}$C-lactate and puromycin were incubated in the same media without cells, with NAD and lactate dehydrogenase, and with boiled cells. No lactyl-puromycin could be detected in these experiments.

## 4. Discussion

From intact cells of *B. subtilis* and *S. cerevisiae* incubated with puromycin and $^{14}$C-lactate, a compound was isolated which is considered to be L-lactyl-puromycin. This is based on the following observations: 1) The compound co-migrates with synthetic lactyl-puromycin upon paper electrophoresis at three different pH's and upon paper chromatography. 2) In double labelling experiments with $^{14}$C-lactate and $^{3}$H-puromycin, both types of label are incorporated. 3) The formation of this compound is dependent on the presence of puromycin and the yield increases as the concentration of antibiotic is raised. 4) Total hydrolysis in 6 N HCl or digestion with Pronase liberates lactic acid, which was identified in different electrophoretic and chromatographic systems. 5) The lactic acid is of the L-configuration, as it was oxidized by L-lactate dehydrogenase in the presence of an NADH-trapping system. The pyruvate formed could further be converted to alanine with pyruvic-glutamic-transaminase. 6) Digestion with Nagarse yields N-lactyl-O-methyl-tyrosine. Further cleavage with carboxypeptidase A liberated lactic acid and O-methyl-tyrosine.

The mechanism by which puromycin inhibits protein biosynthesis has been studied extensively [4,5]. In particular, this antibiotic has been shown to react with the chain initiator N-formyl-methionyl-tRNA, bound to ribosomes, thereby forming N-formyl-methionyl-puromycin [6]. The formation of lactyl-puromycin is, apparently, another example of an abortive reaction induced by puromycin, but the nature of the lactyl-derivative interacting with the antibiotic is at present unknown. It could be that puromycin inhibits some reaction in the metabolism of lactyl-CoA, which has been shown to occur in different system [7,8]. Since lactyl-puromycin is formed in a eukaryotic organism (yeast) as well as in *B. subtilis*, a metabolic reaction of more general importance could be involved.

## Acknowledgements

One of us (K.A.D.) holds a postdoctoral fellowship from the Muscular Dystrophy Association of Canada. This work was supported in part by U.S. Public Health Service Grant R05 TW00198.

## References

[1] H.Bachmayer and G.Kreil, Biochim. Biophys. Acta, in press.
[2] K.Horikoshi and R.H.Doi, Fed. Proc. 26 (1967) 457.
[3] B.Ephrussi and P.P.Slonimski, Biochim. Biophys. Acta 6 (1950) 256.
[4] D.Nathans, Proc. Natl. Acad. Sci. U.S. 51 (1964) 585.
[5] I.Rychlík, Biochim. Biophys. Acta 114 (1966) 425.
[6] M.S.Bretscher and K.A.Marcker, Nature 211 (1966) 380.
[7] P.R.Vagelos, J.M.Earl and E.R.Stadtman, J. Biol. Chem. 234 (1959) 765.
[8] R.Rabin, H.C.Reeves, W.S.Wegener, R.E.Megraw and S.J.Ajl, Science 150 (1965) 1548.