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Note

Radioaffinity labelling of β-D-galactosidase from *Escherichia coli* with [^{14}C]-glycerol, mediated through covalently bound 2,6-anhydro-1-deoxy-D-*galacto*-hept-1-enitol

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The preceding paper[1] describes the application of 2,6:3,4-dianhydro-1-deoxy-D-*talo*-hept-1-enitol (**1**) as a potential affinity label for the β-D-galactosidase from *E. coli*. Compound **1**, although having a reasonable K_i value of 21mM, does not link exclusively to the substrate binding site of the enzyme, but links also to other groups of the protein molecule that have no influence on the catalytic activity. Thus ^{3}H-labelled **1** attaches itself to the tetrameric enzyme in the molar ratio of ~48:1. This chemically modified enzyme, in comparison with an untreated blank, had lost less than 7% of its activity in a colorimetric assay with an *o*-nitrophenyl β-D-galactoside* (niphegal).

Discrimination between true affinity labelling and random labelling may be possible if the covalently attached label at the substrate-binding site would, because of its very presence there, undergo exclusively a secondary reaction catalyzed by the enzyme itself.

RESULTS AND DISCUSSION

2,6-Anhydro-1-deoxy-D-*galacto*-hept-1-enitol (**2**) is converted into 1-deoxy-D-*galacto*-heptulose (**3**), or, in the presence of the acceptor, glycerol[2], into glycerol-1-yl 1-deoxy-β-D-*galacto*-heptulopyranoside (**4**) by β-D-galactosidase in phosphate buffer.

As it could be demonstrated[1] that 2,6:3,4-dianhydro-1-deoxy-D-*talo*-hept-1-enitol (**1**) becomes covalently attached to the substrate-binding site of β-D-galactosidase, probably through its strongly electrophilic C-3 to form **2-E**, there should be no

*Complete loss of activity in the niphegal assay can only be reached when the enzyme has been treated with extremely high concentrations of **1** (>~0.5M). These concentrations also cause exhaustive, random labelling of the protein[1].

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difference between free substrate (**2**) and covalently bound substrate (**2-E**) as far as enzyme-catalyzed addition of glycerol to the double bond is concerned. When β-D-galactosidase is incubated with high concentrations of **1** (0.63M) in a medium also containing [^{14}C]glycerol, the protein becomes covalently ^{14}C-labelled, as could be demonstrated by SDS-electrophoresis on acrylamide gel. Thus 0.13 mol of [^{14}C]-glycerol becomes linked to one mol of tetrameric protein, meaning that 3.2% of the enzyme's substrate-binding sites are labelled*. This result corresponds roughly with a yield of 11% of isolated **4**, as obtained when compound **2** and β-D-galactosidase are incubated for 2 days in the presence of 0.86M 1,3-[^{14}C]glycerol as acceptor[2].

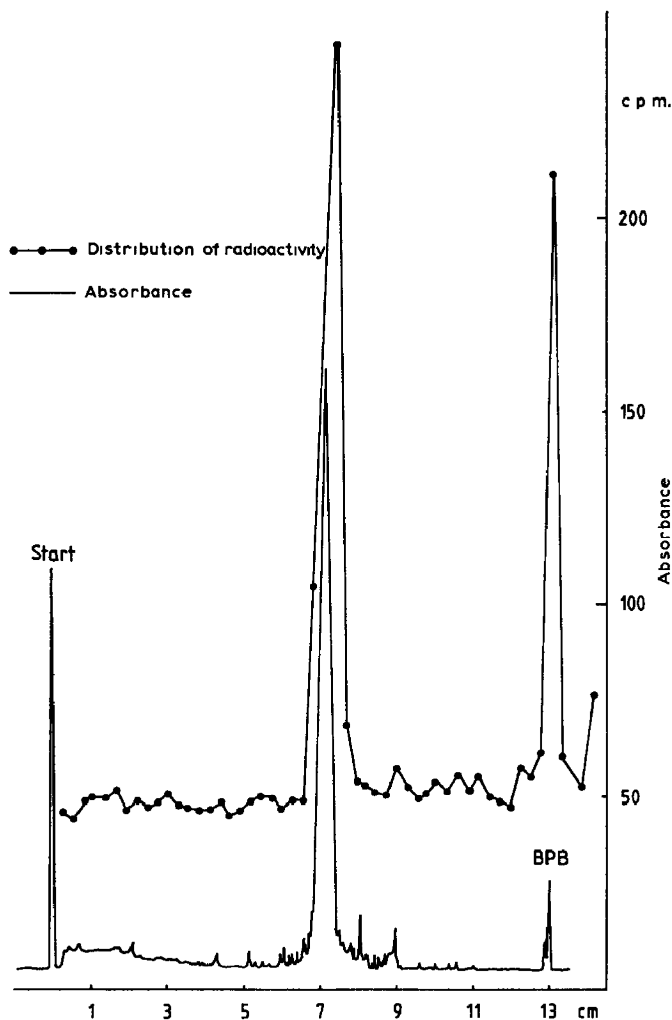
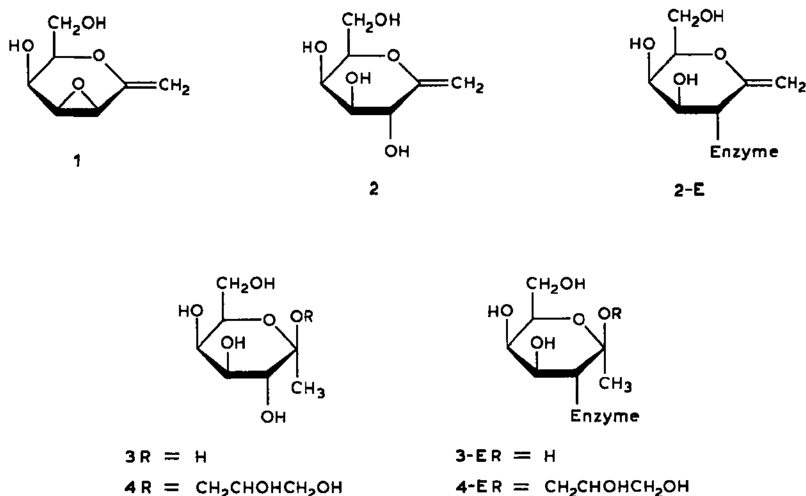


Fig. 1. SDS-Disc electrophoretogram of ^{14}C-labelled β-D-galactosidase.

*The conditions for this labelling experiment are given under "Experimental".



The zone of radioactivity coincides with the protein band detected by staining with Coomassie Blue (Fig. 1). [^{14}C]Glycerol neither binds to the protein in the absence of **1** nor does it react with **1** or its hydrolyzed product **2** in the absence of the enzyme. It is therefore reasonable to assume that labelling of β-D-galactosidase by glycerol is mediated through **1** covalently attached to the active site of the enzyme as **2-E***. As addition of [^{14}C]glycerol to the double bond of the attached heptenitol (**2-E**) is likely to occur in the same way as to heptenitol **2**, added as substrate, this process can only happen at the active site.

EXPERIMENTAL

Materials and methods. — [^{14}C]Glycerol (171 mCi/mmol) in water (5 mL) was purchased from the Radiochemical Centre (Amersham, England)**. Before use, the water was removed by drying the solution in a vacuum desiccator over magnesium perchlorate.

2,6:3,4-Dianhydro-1-deoxy-D-*talo*-hept-1-enitol[5,6] (**1**) was crystallized once

*A similar experiment would be feasible with ^3H_2O instead of [^{14}C]glycerol. Conclusions may be drawn if ^3H_2O from the incubation medium were added to the double bond of 2-E. The expected strong background labelling of the protein through unspecific exchange reactions, as well as a possible large isotopic discrimination in the actual addition reaction, made us choose [^{14}C]glycerol for the purpose. The indirect labelling method with ^3H_2O was tried by Wentworth and Wolfenden[3] and failed to prove the formation of an actual 2-deoxy-D-*lyxo*-hexosyl enzyme-intermediate[4] in the β-D-galactosidase-catalyzed hydration of D-galactal.

**According to J. R. Zabrecky and R. D. Cole[8], [^{14}C]glycerol from the Radiochemical Center (Amersham, England) contains a contaminant that binds nonspecifically to protein. We found that [^{14}C]-glycerol, whether purchased from Amersham or NEN, is converted into an as-yet unknown compound or compounds when exposed to the atmosphere. This contaminant cannot be separated completely from the protein by dialysis. In SDS electrophoresis, however, it migrates together with Bromothymol Blue, and not with the protein.

from acetone and twice from ethyl acetate. Highly purified β-D-galactosidase from *Escherichia coli* (β-D-galactoside galactohydrolase, E.C. 3.2.1.23; Dr. W. Littke, Chem. Lab., Univ. Freiburg, W Germany) had a specific activity of 242 U/mg. A suspension (1 mL) of it in saturated, aqueous ammonium sulphate was centrifuged and the supernatant solution discarded. The precipitate was dissolved in 50mM sodium phosphate buffer (pH 6.8, 0.2 mL) containing magnesium chloride (mM) and dialyzed three times at 4° against 500-mL portions of buffer.

The concentration of protein was determined by the biuret procedure[7] with crystalline bovine serum albumin as standard. Radioactivity (^{14}C) was counted in a multi-user, liquid scintillation counter (BF 815, Berthold). The scintillation cocktails used were: *A*, Unisolve 1 (Zinsser, 6 mL); and *B*, Protosol (NEN, 0.7 mL), water (0.1 mL), and Quickszint 501 (Zinsser, 8 mL). Counting efficiency was determined by the external-standard procedure with a standard quench-correction curve. The discontinuous dodecyl sulphate poly(acrylamide) gel electrophoresis[9] was performed in tubes (6 mm diam., 170 mm length) according to O'Farrell[10]. The total acrylamide concentration of the gels was 5% at a ratio of 97.2:2.8 acrylamide:bis-(acrylamide). The length of the separation gels was 110 mm. Samples of protein (15 μg) were applied to each gel. The temperature was maintained at 10° during the electrophoresis, and the current was kept constant at 1 mA/gel tube. After electrophoresis, one gel was cut into 2-mm slices in which the radioactivity was determined (cocktail *B*). A second gel was fixed with 12.5% trichloroacetic acid and stained with Coomassie Brilliant Blue R 250.

Incubation of β-D-galactosidase from E. coli *with* **1** *and* [^{14}C]*glycerol.* — To [^{14}C]glycerol (213 μCi, 171 mCi/mmol) β-D-galactosidase (16.24 mg in 0.4 mL of buffer) and crystalline **1** (39.11 mg, 0.25 mmol) were added. The micromolar ratio in the solution of substrate **1**, acceptor glycerol, and tetrameric enzyme was thus 625: 1.24:0.035. After 3 days* at room temperature, the incubation mixture (0.4 mL) was divided in halves (0.2 mL each), which were separately dialyzed against buffer until no more radioactivity was released into the dialysate. The radioactivity of the protein solutions was determined by liquid-scintillation counting (cocktail *A*), and accounted for 0.3 μCi of [^{14}C]glycerol in the first half and 0.46 μCi in the second half, which constitutes a total of 0.0044 μmol of [^{14}C]glycerol bound to 0.035 μmol of tetrameric enzyme (0.13 mol/mol). The dialysed protein was examined by SDS-gel electrophoresis as already described.

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*Longer incubation did not seem advisable because, after this time, the enzyme had lost >95% of its catalytic activity[1,6].

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