REDUCTION OF NITRO DERIVATIVES OF 1,2-DIHYDRO-3H-1,4-BENZDIAZEPIN-2-ONES BY IMMOBILIZED CELLS OF *E. coli*

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In the search for more effective and less toxic tranquilizers, it was of interest to synthesize amino-derivatives of 1,2-dihydro-3H-1,4-benzdiazepin-2-ones. This has up to the present time been achieved by reduction with hydrogen in the presence of a catalyst (Adams platinum, palladium, or Raney nickel [6, 14, 15]) of the appropriate nitro compounds. This afforded mixtures of di- and tetrahydro-1,4-benzdiazepin-2-ones, partial reduction products (nitroso, hydroxylamino, and azoxy derivatives), and reduction of halo derivatives resulted in removal of the halogen atom. The separation of these mixtures required the use of special methods [7]. We therefore set out to examine the microbiological reduction of nitro-1,2-dihydro-3H-1,4-benzdiazepin-2-ones to the corresponding amino derivatives.

EXPERIMENTAL

The work was carried out with the following strains of *E. coli*: VKMV-471, 835, and 870 (obtained from the All-Union collection of microorganisms of the Institute of Biochemistry and Physiology of Microorganisms, Academy of Sciences of the USSR).

The medium employed had the following composition (in 100 ml of water): 0.6 g of peptone, 0.6 g Na_2HPO_4, 0.3 g KH_2PO_4, 0.1 g NaCl, 0.001 g $CaCl_2$, and 0.4 g of glucose. Sterilization was carried out at 0.5 atm for 30 min.

The nitro-1,2-dihydro-3H-1,4-benzdiazepin-2-ones were obtained as described in [5], and markers for the appropriate amino compounds by reduction of the nitro compounds with Raney nickel as described in [15].

Thin-layer chromatography was carried out on Silufol UV-254 plates in the following solvent systems: toluene—acetone—ammonia (25:25:0.2), hexane—acetone (1:1), and benzene—propanol—ammonia (180:40:2). UV spectra were obtained on an SF-16 spectrophotometer, IR spectra on a Perkin-Elmer 577, and mass spectra on a Varian MAT-112 with direct sample introduction. Ionizing electron energy was 70 eV.

Microbiological reduction was carried out as follows. *E. coli* cells (strain VKMV-471, 835, or 870) were inoculated on nutrient agar for 18 h at 37°C, washed with 0.8% NaCl, centrifuged at 7000 rpm for 15 min, and washed twice with 0.8%. NaCl (pH 7.0-8.0). The nitro-1,2-dihydro-3H-1,4-benzdiazepin-2-one (0.3-3 mg per 100 ml of buffer solution) in ethanol was added, and the mixture incubated for 4-6 h (at 20 and 37°C). The required product was extracted with organic solvents (chloroform, methylene chloride, light petroleum, or mixtures of these), and the extract evaporated to dryness under reduced pressure. After purification by recrystallization, the amino-1,2-dihydro-3H-1,4-benzodiazepin-2-one was obtained.

To accumulate biomass, cultures of *E. coli* strains VKMV-471, 835, and 870 from nutrient agar were transferred to 100 ml of liquid medium of the composition given above in a flask and incubated on a shaker (200 rpm) at 28°C for 18 h, the nitro-1,4-benzdiazepin-2-one (0.3-3 mg per 100 ml of medium, in alcohol) was added and the mixture cultured for 4-6 h without agitation at pH 7.0-8.0, or the culture of *E. coli* , strain VKMV-471, 835, or 870 from nutrient agar was transferred to a 100-ml flask with 25 ml of liquid medium of the same composition. The contents of the flask were centrifuged at 7000 rpm, washed with 0.8% NaCl, and again centrifuged at 7000 rpm. To the cells thus obtained, in buffer solution (pH 7.0-8.0) was added the nitro-1,4-benzdiazepin-2-one (0.3-3 mg/100 ml of buffer, in alcohol), and

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cultured at 20 and 37°C for 4-6 h, followed by isolation of the desired product by the method described above.

The activities of native *E. coli* cells in the reduction of nitro-1,2-dihydro-3H-1,4-benzdiazepin-2-ones was assessed as described in [1], as the amount of transformation product obtained, expressed as a percentage of the weight of the initial substrate. Values of 60-70% were obtained, or 0.38 μmole/h per 1 g of moist weight of cells.

Immobilization of the cells in polyacrylamide gel (PAAG) was carried out as described in [2]. The polymerization mixture had the following composition: acrylamide 0.475 g, N,N'-methylenebisacylamide 0.025 g in 2.7 ml of potassium phosphate buffer, 7.5 mg of ammonium persulfate in 0.75 ml of distilled water, and 1 drop of N,N,N',N'-tetramethylethylenediamine. The time for formation of the gel was 1-1.5 min. The resulting block of gel was broken up mechanically, passed through a sieve, and washed with fifty times its volume of distilled water. There was obtained 5 g of 10% PAAG with immobilized cells.

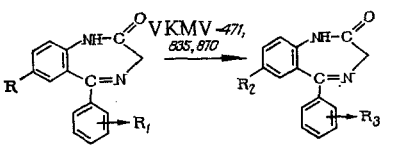
The incubation mixture for the reduction of the nitro-1,2-dihydro-3H-1,4-benzdiazepin-2-ones consisted of 5 g of PAAG with 0.5 g of *E. coli* cells in 8 ml of potassium phosphate buffer and 0.1 ml of the nitro compound in 0.1 ml of ethanol. For comparison, control experiments were carried out with the same amounts of native cells and substrate. Incubation time, 6 h.

The optimum temperature for the reduction with native and immobilized *E. coli* cells was determined under the above conditions at 10, 20, 30, 40 and 50°C.

The keeping qualities of the immobilized *E. coli* cells were checked in water and buffer solution at 4-5°C.

RESULTS AND DISCUSSION

Studies have previously been carried out on the microbiological reduction of nitrobenzene [10], chloramphenicol [12-13], nitrobenzoic acid [17], and nitrofuran [16]. The corresponding amines were obtained, but in yields no greater than 30-40%. The microbiological transformation of nitro-1,2-dihydro-3H-1,4-benzdiazepin-2-ones by *E. coli* strains VKMV-471, 835, and 870 afforded products, the physicochemical constants of which indicate the formation of the amino-1,2-dihydro-3H-1,4-benzdiazepin-2-ones, as follows:



I-X XI-XIII

	R	R_1		R_2	R_3
I	NO_2	H	XI	NH_2	H
II	H	m-NO_2	XII	H	m-NH_2
III	H	p-NO_2	XIII	H	p-NH_2
IV	CH_3	m-NO_2	XIV	CH_3	m-NH_2
V	CH_3	p-NO_2	XV	CH_3	p-NH_2
VI	Cl	m-NO_2	XVI	Cl	m-NH_2
VII	Cl	p-NO_2	XVII	Cl	p-NH_2
VIII	Br	m-NO_2	XVIII	Br	m-NH_2
IX	Br	p-NO_2	XIX	Br	p-NH_2
X	NO_2	o-Cl	XX	NH_2	o-Cl

The UV spectrum of (XI) thus exhibits absorption with λ_{max} 240 and 360 nm (ε $mole^{-1}$-cm^{-1} 4.42 and 3.39).

The mass spectrum of the compound confirmed the molecular mass as 251, the spectrum displaying the following characteristic peaks: m/e 252 (11.1), 251 (100), 250 (21.2), 223 (49.3), 222 (50.1), 69 (5.0).

TLC using several solvent systems showed that the product of microbiological synthesis was fully identical with 7-amino-1,2-dihydro-3H-1,4-benzdiazepin-2-one obtained by a chemical route.

TABLE 1. Yields of (XIV) at Various pH Values (as % of maximum)

pH	Yield of compound
6,8	85
7,0	96
7,2	100
7,4	100
7,6	100
7,8	100
8,0	93
8,2	82

TABLE 2. Effect of PAAG on the Reduction of Nitrazepam (I) by Cells Immobilized in PAAG (0.2 g of cells in 5 g of PAAG).

Expt. No.	Expti. condition	Yield of 7-amino compound as a percentage of the max
1	Free cells E. Coli	100
2	Free cells E. Coli + PAAG, temp. 37°, incubation time 12 h	49,1
3	Free cells E. Coli + PAAG, temp. 20°, incubation time 6 h	77,4
4	Cells of E. Coli, immobilized in PAAG, temp. 37°, incubation time 12 h	44,7
5	Cells of E. Coli, immobilized in PAAG, temp. 20°, incubation time 6 h	53,9
6	Cells of E. Coli, immobilized in PAAG, temp. 20°, incubation time 6 h, 0.5 g of cells in 5 g of PAAG	86,2

The yields of (XI) and the other amino-derivatives were high (60-70%), and no tetrahydro-1,4-benzdiazepin-2-ones were formed.

The best yields of amino-1,2-dihydro-3H-1,4-benzdiazepin-2-ones were obtained using a buffer solution or a nutrient medium of pH 7.0-8.0. Table 1 presents data showing the dependence of the extent of conversion of (XIV) on the pH of the buffer solution.

The nitro-1,2-dihydro-3H-1,4-benzdiazepin-2-ones were introduced in a concentration of 0.3-3 mg per 100 ml of nutrient medium. Concentrations lower than this were undesirable, since yields were then reduced. On examination, 7-amino-1,2-dihydro-3H-1,4-benzdiazepin-2-one was found to have no effect on the nitroreductase activity of *E. coli* cells, but increasing the concentration of 7-nitro-1,2-dihydro-3H-1,4-benzdiazepin-2-one above 3 mg per 100 of nutrient solution reduced the yield of the 7-amino derivative by 15-20%.

Since, in this instance, the nitroreductase is an intracellular enzyme, which can only be isolated with difficulty, and the substrate and required products are not macromolecular compounds, it was of interest from the practical point of view to examine reduction by *E. coli* cells, immobilized in PAAG. The nitroreductase activity of immobilized *E. coli* cells has not hitherto been studied, but there are reports of the enzymic preparation of l-tryptophan [4], 6-aminopenicillanic acid [11], L-asparaginic acid [8], and glutathione [9], and the possibilities of PAAG, carrageenin and gelatin gels, and copolymers of styrene and divinylbenzene, activated by sepharose and metal (Ti^{4+} and Zr^{4+}) hydrogels, have been considered. More often than not, PAAG is used in biotechnological processes. However, as has previously been observed [3] when microbial cells are incorporated into PAAG their relative viability is greatly reduced, and in the present case the effect of prepared PAAG on the ability to *E. coli* cells to reduce nitro-1,4-benzdiazepin-2-ones has been checked (Table 2).

The nitroreductase activity of immobilized *E. coli* cells was compared with that of the free cells, which was 0.38 μmole per 1 g of cells.

It will be seen from the data presented in Table 2 that PAAG has a strong inhibitory effect at a temperature of 37°C and incubation time 12 h (up to 50.9%), whereas at 20°C and an incubation time of 6 h 77.4% of (XI) was obtained. A reduction in the nitroreductase activity of the cells when immobilized in PAAG was also observed, this being greater at a

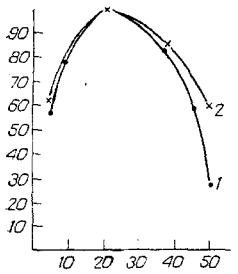


Fig. 1. Plots of the extent of conversion of 7-nitro-1,2-dihydro-3H-1,4-benzdiazepin-2-one by *E. coli* cells against temperature. 1) *E. coli* cells immobilized in PAAG; 2) free *E. coli* cells. Horizontal axis, temperature (°C); vertical axis, yield of 7-amino-1,2-dihydro-3H-1,4-benzdiazepin-2-one (as a percentage of the maximum).

temperature of 37°C, incubation time 12 h, and with a smaller quantity of cells (0.2 g). On the assumption, as in [3], that the inhibitory effect of PAAG on the ability of *E. coli* cells to effect reduction is due to the toxic effects of residual acrylamide monomer, we examined the effects of various concentrations of acrylamide (temperature 20°C, incubation time 6 h). The greatest amount of the desired product (XI) was formed using 5% of acrylamide, 10% and 15% of acrylmide causing 23-26% inhibition as compared with the 5% gel.

The maximum amount of *E. coli* cells in 5 g of PAAG was 1.5 g (moist weight), but even with 0.5 g of cells the yield of (XI) was 86.2% of the yield when the same amount of free cells was used, and with 1 g and 1.5 g this level was maintained (86.6 and 87.7% respectively), although the mechanical properties of the PAAG were adversely affected, resulting in the liberation of cells into the buffer solution following repeated use.

Since, in this instance, the appropriate nitroreductase is an intracellular enzyme which is difficult to isolate, and the substrate and the desired products are not macromolecular compounds, it was of interest from the practical point of view to examine reduction by *E. coli* cells immobilized in PAG (duration of experiment, 6 h). Figure 1 shows that both for the free cells, and those immobilized in polyacrylamide gel, the highest yields of amino-1,4-benzdiazepines were obtained at 20°C; raising the temperature to 50°C led to a sharp decrease in the activity of the immobilized cells (to 27.1% of the maximum yield) and of the free cells (to 59.3%), apparently as a result of the toxic effects of the components of the PAG. The dependence of the PAG-immobilized *E. coli* cells on pH was the same as for the free cells.

Tests of the possibility of repeated use of the immobilized *E. coli* cells in a batch reactor showed that the highest yields of amino derivatives were obtained in the second run. When used for a third time, the activity was 28.9% of the maximum value, but it then remained approximately at this level for fifteen stages, and after storing this catalyst for a month in a buffer at 4-5°C, its activity was 19% of its initial value.

Tests of the storage life of immobilized *E. coli* cells at 4-5°C showed that after three months the activity of the immobilized cells was 29% in buffer, and 32% of the initial value in water.

These results therefore show that the microbiological reduction of nitro-1,2-dihydro-3H-1,4-benzdiazepin-2-ones by *E. coli* cells can be used to synthesize the corresponding amino-derivatives in 60-70% yields, and that when *E. coli* cells are immobilized in PAG they retain 80% of their original activity.

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