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EXPERIMENTAL ARTICLES

Manganese-Dependent Ribonucleotide Reductase of *Propionibacterium freudenreichii* subsp. *shermanii*: Partial Purification, Characterization, and Role in DNA Biosynthesis

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Abstract—Like *Lactobacillus leichmanii, Rhizobium meliloti*, and *Euglena gracilis, P. freudenreichii* implicates cobalamin in DNA anabolism via adenosylcobalamin-dependent ribonucleotide reductase. However, in the absence of corrinoids, *P. freudenreichii* is able to synthesize DNA with the involvement of an alternative ribonucleotide reductase, which is independent of adenosylcobalamin. This enzyme is localized in both the cytoplasm (80% of activity) and the cytoplasmic membrane (20% of activity), being loosely bound to the latter. Experiments with partially purified ribonucleotide reductase isolated from extracts of corrinoid-deficient cells showed that manganese specifically stimulates this enzyme and that it is composed of two protein components, a feature that is typical of all metal-containing reductases activated by molecular oxygen. Low concentrations of manganese ions enhanced DNA synthesis in corrinoid-deficient manganese-limited cells. This effect was prevented by the addition of 80 mM hydroxyurea, a specific inhibitor of metal-containing aerobic ribonucleotide reductases. It was concluded that, in adenosylcobalamin-deficient *P. freudenreichii* cells, DNA synthesis is provided with deoxyribosyl precursors through the functioning of manganese-dependent aerobic ribonucleotide reductase composed of two subunits.

Key words: Propionibacterium freudenreichii, ribonucleotide reductase, manganese, adenosylcobalamin, DNA biosynthesis.

Propionibacterium freudenreichii subsp. *shermanii* VKM B-103, which normally synthesizes high amounts of corrinoids, implicates adenosylcobalamin (AdoCbl) in DNA synthesis via AdoCbl-dependent ribonucleotide reductase [1–5]. In most organisms, ribonucleotide reductase (RNRase, EC 1.17.4.1) catalyzes the formation of deoxyribosyl precursors; hence, it is the primary specific enzyme of DNA synthesis. Since the formation of deoxyribonucleotides is the rate-limiting stage of DNA synthesis, the physiological role of ribonucleotide reductase is to control the DNA formation rate [6–8].

Prokaryotic RNRases are structurally diverse. Representatives of the domains *Bacteria* and *Archaea* contain five types of RNRase differing in their coenzyme moiety. With regard to their response to molecular oxygen, there exist O_2-insensitive, O_2-activated, and O_2-inactivated RNRases [9]. The predominant type of bacterial RNRases is AdoCbl-dependent RNRase insensitive to O_2 and to hydroxyurea (HU). The enzyme is either monomeric with M_r = 72–90 kDa or homodi(tetra)meric protein [10–12]. Metal-containing RNRases with nonheme iron or manganese in their active center, which are activated by oxygen and inhibited by HU, are heterodimers composed of the large catalytic subunit R1 and the small metal-containing coenzyme subunit R2 [13]. The RNRases of *Corynebacterium ammoniagenes* are heterodimers composed of catalytic (R1) and coenzyme (R2) subunits close in size (about 100 kDa) [14, 15]. Fe-containing RNRases were revealed in representatives of *Enterobacteriaceae* and in *Lactococcus lactis* [13, 16], whereas Mn-containing enzymes were revealed in some coryneforms [14, 15].

P. freudenreichii contains two RNRases differing with respect to AdoCbl. When corrinoids are not synthesized, the bacterium retains its ability to synthesize DNA, to grow, and to divide, since one of its RNRases (namely, aerobic RNRase) does not require AdoCbl. The functional and kinetic parameters of this enzyme, which is usually designated as alt-RNRase, considerably differ from those of AdoCbl-dependent O_2-insensitive RNRase functioning in corrinoid-sufficient *P. freudenreichii* cells [2, 3, 5].

The aim of the present work was to isolate and partially purify alt-RNRase, to investigate the requirement of this enzyme for Mn^{2+} ions, to study its structural properties, and to elucidate the effect of manganese on DNA synthesis in corrinoid-deficient *P. freudenreichii* cells.

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MATERIALS AND METHODS

Corrinoid-deficient (Cor$^-$) cells of *Propionibacterium freudenreichii* subsp. *shermanii* VKM B-103 were obtained by aerobic batch cultivation (without shaking) in a cobalt-free medium [5] supplemented with 0.1% peptone. Cells were harvested in the mid-exponential phase (65–68 h of growth). The residual content of corrinoids in such cells was 1–2 μg/g dry wt as compared to 1000 μg/g dry wt in corrinoid-sufficient cells. Manganese-limited cells were obtained by successively subculturing them three times in the aforementioned medium without the addition of any manganese salt.

Washed cells were suspended, in a proportion of 1 : 2 (w/v), in buffer A (70 mM Tris–HCl, pH 7.9–8.0) supplemented with 2 mM dithiothreitol (DTT) and disrupted by sonication in a UZDN-2T disintegrator (Russia) at 22 kHz, 40 μA, 0°C for a total of 200 s in 20-s bursts with 2-min breaks for cooling. The homogenate was centrifuged at 30000 *g* for 20 min (6°C) to remove debris, and the supernatant liquid was dialyzed against buffer A. The dialyzed cell extract was used for the isolation of alt-RNRase and the determination of its cellular location by differential centrifugation.

The enzyme was assayed at 37°C in buffer A supplemented with 2.5 mM ADP and 24 mM DTT. The reaction mixture had a volume of 200 μl; incubation time was 60 min. The concentration of the deoxyribosylated product of reaction was determined either with diphenylamine [10] or by the radiochromatographic HPLC method [5].

Before the enzyme isolation, the dialyzed cell extract was treated with a solution of streptomycin sulfate (Str-SO_4) in buffer A to remove nucleic acids and ribosomes [14]. The final concentration of Str-SO_4 in the cell extract was 1.5%; treatment was carried out at 0–6°C for 40 min with occasional gentle stirring. After treatment, the precipitate was removed by centrifugation at 5000 *g* for 15 min (6°C), and the supernatant liquid was dialyzed at 6°C for 16 h against buffer A in a proportion of 1 : 100.

The proteins of the dialyzate were fractionated by anion-exchange chromatography on DEAE-cellulose (Whatman 52) carried out in batch mode. Proteins were eluted with a stepwise KCl gradient in buffer A. The active protein fraction eluted with 0.38 M KCl was concentrated on PM 30 or PM 10 Amicon membranes and rechromatographed on a Mono Q HR 5/5 anion-exchange column (Pharmacia–LKB, Sweden) using a linear KCl gradient at a flow rate of 1 ml/min (1.5 MPa; 20°C). The same column was used for the dissociation of purified alt-RNRase into subunits in a chromatographic buffer lacking Mg^{2+} ions.

The molecular mass of alt-RNRase was estimated by gel filtration [20] on a Superose 6 HR 10/30 column (Pharmacia–LKB) using the following protein markers: cytochrome *c* (12.4 kDa), RNase (14 kDa), soybean trypsin inhibitor (21.5 kDa), ovalbumin (45 kDa),

Table 1. Localization of alt-RNRase in corrinoid-deficient *P. freudenreichii* cells

Cell fraction	Total enzyme activity		Protein		Specific enzyme activity, nmol/(mg protein h)
	nmol/(ml h)	%	mg/ml	%	
Cell homogenate cleared by centrifugation at 30000 *g*	225.0	100.0	7.2	100.0	31.25
Soluble fraction (supernatant after centrifugation at 144000 *g*)	176.0	78.0	5.8	80.5	30.34
Structural cell components (CPM + ribosomes)	42.6	18.9	1.1	15.3	38.73

Note: Cell homogenate was obtained by the extrusion of frozen cells in a Hughes press. The volumes of all fractions were equal. CPM is cytoplasmic membrane.

Table 2. Batch fractionation of streptomycin-treated extract of *P. freudenreichii* cells on DEAE-cellulose 52

Step	Total enzyme activity, nmol/(ml h)	Protein, mg/ml	Specific enzyme activity, nmol/(mg protein h)	Purification factor
Cell extract	202	8.00	25.25	1
Cell extract treated with Str-SO_4	197	7.60	25.92	
0.20 M KCl (~0.15 M)	0	1.80	0	
0.30 M KCl (~0.26 M)	0	2.63	0	
0.35 M KCl (~0.32 M)	6	0.90	6.70	
0.40 M KCl (~0.38 M)	186	0.60	310.00	12
0.60 M KCl (~0.55 M)	2	0.40	5.00	

bovine serum albumin (BSA, 67 kDa), and BSA dimer (129 kDa). Gel filtration was carried out at room temperature at a flow rate of 0.2 ml/min; the fraction volume was 0.8 ml. The molecular mass (Mr) of proteins was determined from a calibration curve plotted as log Mr versus K_{AV}. The partition coefficient, K_{AV}, was calculated by the formula $K_{AV} = (V_E - V_O) : (V_T - V_O)$, where V_E is the elution volume of the protein, V_O is the void column volume estimated as the elution volume of blue dextran, and V_T is the total volume of the gel bed [17].

DNA synthesis in cells was estimated by the incorporation of [8-^{14}C]-adenine (10.2×10^9 Bq/g) into the alkali-stable acid-insoluble cellular fraction. Labeled adenine (74000 Bq/ml) and unlabeled adenine (5 μg/ml) were added to a suspension containing 1.5–2.0 mg dry wt/ml exponential-phase cells, which were preliminarily washed and suspended in cold 0.05% KH_2PO_4 supplemented with 0.5% glucose. The cell suspension was incubated at 37°C for 60 min. Aliquots for analysis were taken every 15 min. The radioactivity of the dried DNA residue on Synpor filters no. 6 (Czech Republic) was quantified in an LKB scintillation counter (Sweden).

DTT, ADP, soybean trypsin inhibitor, and ovalbumin were purchased from Reanal (Hungary), cytochrome c was from Sigma (United States), BSA and BSA dimer were from Serva (Germany), and [8-^{14}C]-adenine was produced in Russia.

RESULTS AND DISCUSSION

For the successful isolation of alt-RNRase, it was necessary to know the cell localization of this enzyme. Bacterial RNRases are either cytoplasmic or membrane enzymes [18, 19]. Unlike animal RNRases, which are strongly bound to the nuclear membrane, bacterial RNRases are loosely bound to the cytoplasmic membrane (CPM) [8]. According to our early estimations, about 20% of the AdoCbl-dependent RNRase of the corrinoid-sufficient cells of propionic acid bacteria is bound to the structural elements of cells (but not to ribosomes [2]), and 80% of this enzyme occurs in the soluble cell fraction [20].

The distribution of alt-RNRase between two fractions—the precipitate obtained by the centrifugation at 144000 *g* of the extract of corrinoid-deficient *P. freudenreichii* cells (this precipitate must contain ribosomes and fragments of the CPM and cell wall [21]) and the supernatant liquid (Table 1)—showed that about 20% of alt-RNRase occurs in the precipitate fraction. Since there are no indications that RNRases can bind to ribosomes or to the cell wall, the aforementioned distribution of alt-RNRase suggests that 20% of it is bound to the CPM.

In another set of experiments, CPM fragments partially bound to DNA were precipitated from the cell-free extract by centrifugation at 82000 *g*. The RNRase activity of the precipitate obtained thus was 19.5–21.0 nmol/(ml h). The treatment of this precipitate with saline solutions containing from 0.2 to 0.6 M KCl (30-min incubation at 6°C under stirring) with the subsequent precipitation of membrane fragments at 100000 *g* showed that 0.35 M KCl almost completely solubilized alt-RNRase: after such treatment, the RNRase activity of the 100000 *g* supernatant was 18.8 nmol/(ml h), whereas membrane fragments lost their activity. These data suggest that alt-RNRase is only loosely bound to the CPM.

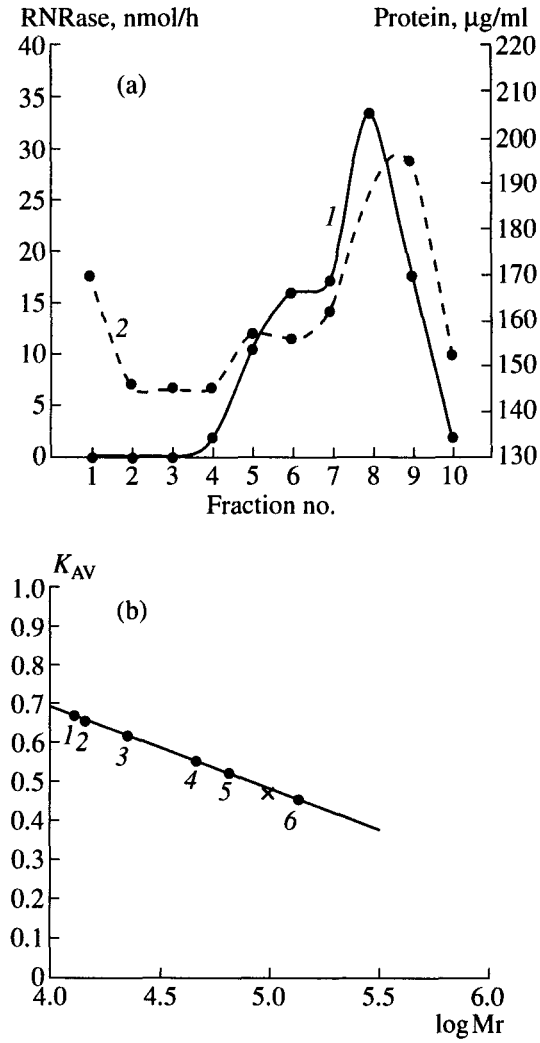

Fig. 1. Estimation of the molecular mass of alt-RNRase by gel filtration. Panel (a): *1*, RNRase activity; *2*, protein concentration. Panel (b): *1*, cytochrome *c* (12.4 kDa); *2*, RNRase (14 kDa); *3*, soybean trypsin inhibitor (21.5 kDa); *4*, ovalbumin (45 kDa); *5*, BSA (67 kDa); *6*, BSA dimer (129 kDa); × denotes the position of alt-RNRase.

Before the isolation of alt-RNRase from the extract of corrinoid-deficient cells, the extract was treated with a solution of Str-SO_4 in buffer A to remove nucleic acids and ribosomes [14, 22]. Fractionation by anion-exchange chromatography on DEAE-cellulose (Whatman 52) carried out in batch mode allowed the protein fraction with RNRase activity to be obtained. The

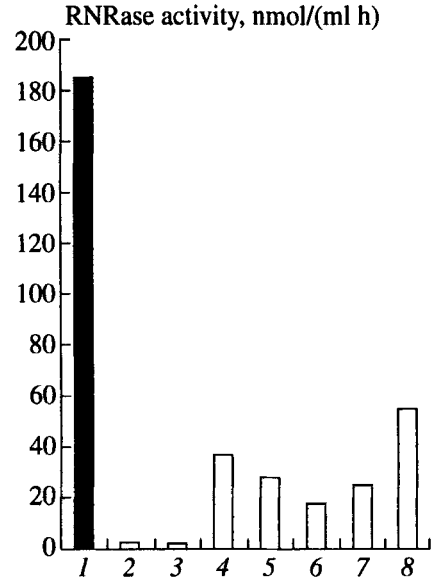

Fig. 2. Activity of the alt-RNRase of *P. freudenreichii* before and after chromatography on a Mono Q column and the effect of mixing of two chromatographic fractions, a and b. *1*, original preparation; *2*, fraction a; *3*, fraction b; *4–8*, fractions a and b mixed in different proportions (proteins, μg/ml): *4*, a + b = 130 + 10; *5*, a + b = 110 + 20; *6*, a + b = 75 + 37; *7*, a + b = 37 + 56; *8*, a + b = 20 + 66.

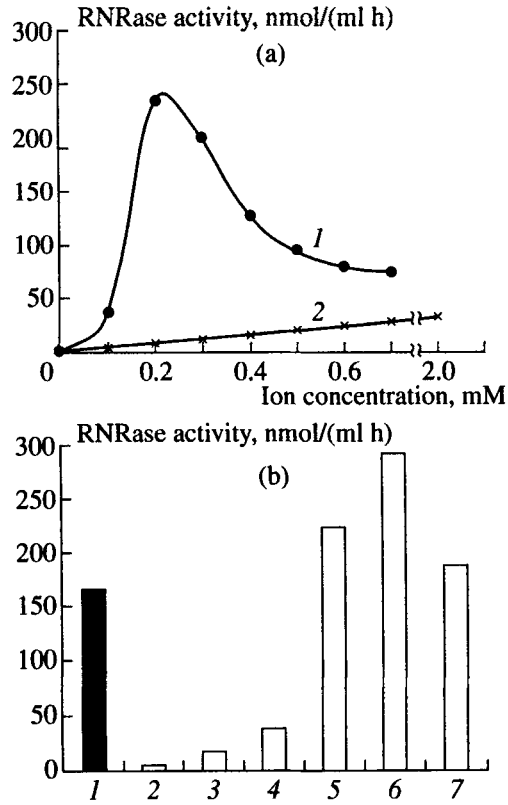

Fig. 3. Activity of the EDTA-treated alt-RNRase of *P. freudenreichii* (Cor^-) in response to (a) separate addition of (*1*) manganese and (*2*) magnesium ions and to (b) combined addition of these ions. Panel b: *1*, before EDTA treatment; *2*, after EDTA treatment; *3*, 0.4 mM Mg^{2+}; *4*, 2 mM Mg^{2+}; *5*, 0.2 mM Mn^{2+}; *6*, 0.2 mM Mn^{2+} + 0.4 mM Mg^{2+}; *7*, 0.2 mM Mn^{2+} + 2 mM Mg^{2+}.

degree of enzyme purification at this stage was 12 (Table 2).

The anion-exchange chromatography of this fraction on a Mono Q column led to a 36-fold purification of alt-RNRase, although most of its activity was lost. Taking into account that metal-containing RNRases composed of two subunits are difficult to isolate in the native form because of their dissociation into subunits [8, 16, 23], further experiments were carried out with the enzyme preparation purified 12-fold by the batch procedure (Table 2, fraction "0.38 M KCl").

The molecular mass of the holoenzyme determined by gel filtration on a Superose 6 column using a calibration curve (Fig. 1) was found to be 96–100 kDa. The presence of a shoulder in front of the main peak of activity indicated that the holoenzyme partially dissociated during gel filtration.

The behavior of the Mn-containing two-component RNRase of *C. ammoniagenes* during gel filtration (dissociation into subunits with close molecular masses even in the presence of Mg^{2+} and reassociation of the subunits into the active enzyme after gel filtration [14, 15]) led us to the assumption that the alt-RNRase of *P. freudenreichii* is also composed of at least two subunits close in molecular mass. It should be noted that, unlike RNRases containing nonheme iron, which cannot be isolated as holoenzymes, the Mn-containing RNRase of *C. ammoniagenes* retained about 50% of its activity during ion-exchange chromatography in the presence of Mg [14].

Magnesium from fraction "0.38 M KCl" was removed by twice-repeated dialysis against Mg^{2+}-free buffer A. The preparation was concentrated and applied to the Mono Q column. Proteins were eluted with a stepwise gradient of KCl (0.2, 0.32, and 0.5 M) in Mg-free buffer. Fractions "0.2 M KCl" (a) and "0.5 M KCl" (b) were concentrated threefold, dialyzed twice, and analyzed for RNRase activity either separately or after their mixing in different proportions in one and the same volume (Fig. 2). It can be seen that separately taken fractions a and b were inactive, but after pooling, they possessed RNRase activity. As is evident from the data presented in Figs. 1 and 2, the AdoCbl-independent alt-RNRase of *P. freudenreichii* is composed of at least two protein components.

Earlier, we showed that alt-RNRase is activated by Mg^{2+}, inhibited by HU, and implicates molecular oxygen in the process of catalysis [5]. Like other aerobic metal-containing RNRases, the alt-RNRase of *P. freudenreichii* is presumably composed of two nonidentical subunits (R1 and R2). The R2 subunit of such RNRases may act as a coenzyme, since it contains catalytically active metal ions (Fe^{3+} or Mn^{2+}).

To investigate the specificity of the alt-RNRase of *P. freudenreichii* (Cor^-) for manganese, the partially purified enzyme was dialyzed against buffer A supplemented with 1 mM EDTA (to remove metal ions) and then against buffer A without EDTA (to remove this

chelating agent from the enzyme preparation). RNRase activity was assayed before and after treatment with EDTA, as well as in the absence and presence of various concentrations of Mg^{2+}, Mn^{2+}, Fe^{2+}, Fe^{3+}, and Co^{2+}. The stock solutions of the respective salts (Mg acetate, $MnCl_2 \cdot 4H_2O$, $FeSO_4 \cdot 7H_2O$, $FeCl_3 \cdot 6H_2O$, and $CoCl_2 \cdot 6H_2O$) were prepared using bidistilled water.

EDTA treatment inactivated alt-RNRase; however, its activity could be restored by Mg^{2+} and Mn^{2+} ions (Fig. 3a and 3b). Co^{2+} at concentrations of 0.1 to 0.4 mM and Fe^{3+} at concentrations of 0.05 to 0.2 mM were ineffective. The addition of Fe^{2+} partially (by 6–7%) reactivated the enzyme; the degree of reactivation increased to 10–12% after the subsequent addition of 0.4 mM Mn^{2+}. The maximum reactivation effect (by 130–148%) was observed after the addition of 0.2 mM Mg^{2+} alone at concentrations of up to 2 mM reactivated the enzyme by no more than 22% (Fig. 3a). The simultaneous addition of 0.2 mM Mn^{2+} and 0.4 mM Mg^{2+} enhanced the activity of alt-RNRase by about 80%, but this stimulating effect decreased at higher concentrations of magnesium ions (Fig. 3b). Therefore, the alt-RNRase of *P. freudenreichii* is probably specific for manganese, while magnesium may promote the interaction (association) of enzyme subunits.

In this connection, of much interest is the effect of manganese on the growth of the manganese-limited *P. freudenreichii* (Cor^-) culture and the total synthesis of DNA. In a medium without Mn, the growth rate of the (Cor^-) culture was 1.5–2 times lower than in the presence of this element. The addition of Mn^{2+} and/or HU to a cell suspension with the subsequent 10-min incubation at room temperature influenced the intensity of DNA synthesis (Figs. 4a and 4b). At relatively low concentrations of Mn^{2+} ions (1.5–3.0 μM), they stimulated DNA synthesis. However, the effect of higher Mn^{2+} concentrations was first stimulating and then inhibiting (Fig. 4a). Presumably, high manganese concentrations unfavorably affected the activity of both RNRase (Fig. 3a) and DNA–DNA polymerases. It should be noted that the effect of manganese on DNA synthesis was weak; nevertheless, it was reproduced in seven independent experiments.

Hydroxyurea, a specific noncompetitive inhibitor of aerobic metal-containing (including Mn-containing) RNRases [14, 15], prevented the stimulating effect of manganese on DNA synthesis in *P. freudenreichii* (Cor^-) cells (Fig. 4a) and inhibited it in the control culture (Fig. 4b). This can be accounted for by the fact that the residual activity of RNRase in Mn-limited cells is sufficiently high to support DNA synthesis (curves *1* and *7* in Fig. 4b) and culture growth.

Thus, the experimental data presented in this paper explain the ability of *P. freudenreichii* subsp. *shermanii* cells to synthesize DNA and to grow in the absence of corrinoids. This ability is due to the presence in these cells of a cobalamin-independent ribonucleotide reductase, a two-component enzyme specific to manganese ions.

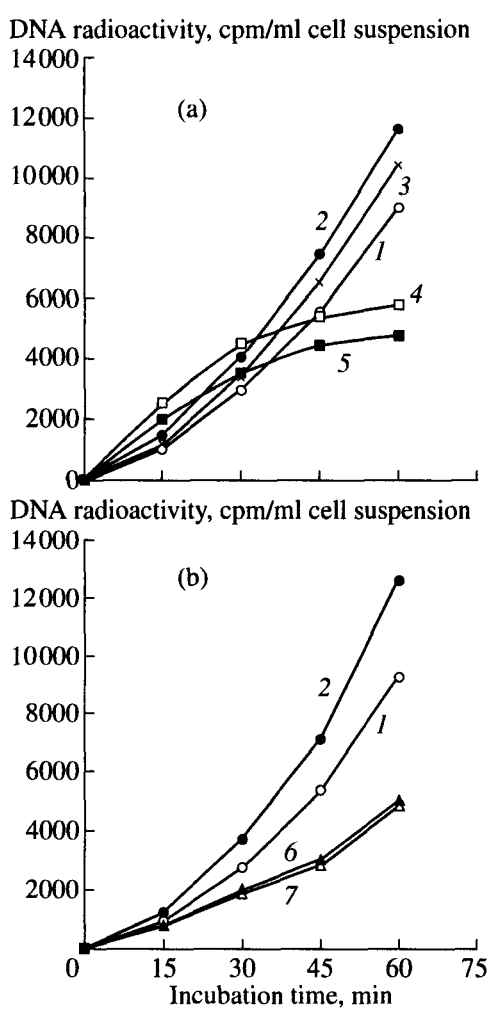

Fig. 4. Effect of manganese ions and hydroxyurea on DNA synthesis in (a) corrinoid-deficient manganese-limited and (b) normal *P. freudenreichii* cells: *1*, without the addition of Mn^{2+} and HU; *2*, 1.5 μM Mn^{2+}; *3*, 3 μM Mn^{2+}; *4*, 5 μM Mn^{2+}; *5*, 10 μM Mn^{2+}; *6*, 1.5 μM Mn^{2+} + 80 μM HU; and *7*, 80 μM HU.

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