Microbiology, Vol. 69, No. 2, 2000, pp. 180–185. Translated from Mikrobiologiya, Vol. 69, No. 2, 2000, pp. 224–230.

EXPERIMENTAL ARTICLES

Stabilization of Enzymes by Dormancy Autoinducers as a Possible Mechanism of Resistance of Resting Microbial Forms

A. I. Kolpakov, O. N. Il'inskaya**, M. M. Bespalov*, F. G. Kupriyanova-Ashina**, V. F. Gal'chenko*, B. I. Kurganov***, and G. I. El'-Registan*[1]**

**Institute of Microbiology, Russian Academy of Sciences, pr. 60-letiya Oktyabrya 7, k. 2, Moscow, 117811 Russia*

***Kazan State University, ul. Kremlevskaya 18, Kazan, 420008 Russia*

****Bach Institute of Biochemistry, Russian Academy of Sciences, Leninskii pr. 33, Moscow, 117071 Russia*

Received July 12, 1999

Abstract—Alkyl-substituted hydroxybenzenes (AHBs), autoinducers of microbial dormancy (or d_1 factors), were found to stabilize the structure of protein macromolecules, making them metabolically less active and more resistant to stresses. In vitro experiments with the *Bacillus intermedius* ribonuclease and chymotrypsin showed that the degree of the physical and chemical stability of these enzymes treated with AHBs depends on their concentration and incubation time. Experiments with RNase, which is capable of refolding, i.e., renaturation after heat denaturation, revealed that AHBs efficiently interact with both intact and denatured proteins. The data obtained allow the inference to be made that d_1 factors may play the role of natural chemical chaperons, blocking metabolism in dormant cells through the formation of catalytically inactive thermostable complexes with enzymes.

Key words: dormancy, dormancy autoinducers, metabolic block, thermostability of enzymes, d_1 factors of microorganisms, chemical chaperons, alkyl-substituted hydroxybenzenes

It has recently been shown that autoregulatory d_1 factors, accumulating in the medium to a threshold level, induce the transition of vegetative microbial cells to a dormant state [1, 2]. In their chemical nature, the known d_1 factors represent mixtures of the isomers and homologues of alkyl-substituted hydroxybenzenes (AHBs) [3, 4]. The mechanism of action of d_1 factors during the development of a metabolically quiescent state is twofold. First, they interact with membrane lipid, causing the crystallization of the membrane lipid stroma, inhibition of the functional activity of membranes (including membrane-associated energy-producing processes), an increase in membrane permeability to monovalent ions, and dehydration of the cell protoplast [5, 6]. Second, AHBs function as low-molecular-weight protein modifiers inhibiting the catalytic activity of enzymes (including nucleic acid depolymerases) and providing for the survival of dormant cells in the absence of functionally active energy-producing processes. The inactivation of enzymes by AHBs has been demonstrated in experiments with chymotrypsin, trypsin, RNase, invertase, and glucose oxidase [7]. The inhibition of metabolism by AHBs is associated with an enhanced resistance of resting cells to various stress factors; therefore, it can be anticipated that AHBs may enhance the stability of protein molecules.

In view of the foregoing, the aim of the present work was to study the stabilizing effect of d_1 factors on enzymes as one of the possible mechanisms of resistance of dormant cells to stresses.

MATERIALS AND METHODS

Experiments on the structural modification of enzymes were carried out using C_6-AHB, an amphiphilic compound with $pK_a = 9$. C_6-AHB (hereinafter, also referred to as the d_1 factor) was added to reaction mixtures in the form of an ethanol solution to give the final ethanol concentration of no more than 5 vol % (in control experiments, equivalent amounts of ethanol were added).

Taking into account that RNases play an important role in the functioning of the hereditary apparatus of cells, are involved in trophic chains, and serve as the extracellular autoregulators of physiological activity, experiments were primarily performed with the RNase (EC 3.1.27.5) of *Bacillus intermedius* purified to an apparent homogeneity (M_r = 12300 Da) [8]. Some

[1]E-mail for correspondence: andlm@mail.ru

0026-2617/00/6902-0180$25.00

Table 1. Effect of C_6-AHB on RNase activity at different pH values

C_6-AHB concentration, %	RNase activity					
	pH 8.5		pH 7.0		pH 6.0	
	(U/mg protein) $\times 10^{-5}$	%	(U/mg protein) $\times 10^{-5}$	%	(U/mg protein) $\times 10^{-5}$	%
0	8.56	100	2.70	100.0	0.74	100.0
0.03	8.50	99.3	2.56	94.8	0.73	98.6
0.05	7.64	89.3	1.61	59.6	0.39	52.7
0.08	2.17	25.4	0.90	33.3	0.30	40.5
0.1	0.86	10.0	0.58	21.5	0.15	20.3

Table 2. Dependence of RNase activity on 15-min preincubation of 0.05% C_6-AHB with different components of the reaction mixture

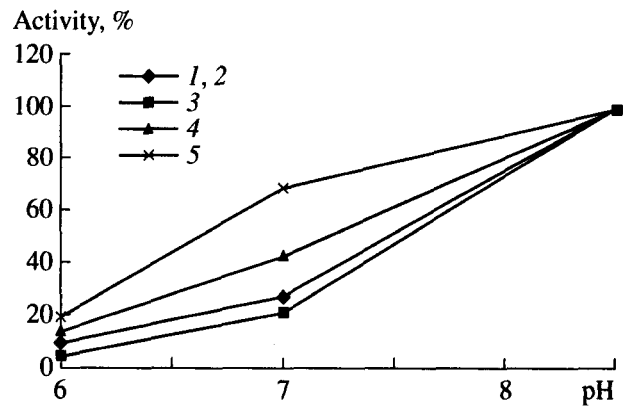
Variant of mixing of reaction components	RNase activity	
	(U/mg protein) $\times 10^{-5}$	%
RNase + buffer [incubation] + RNA + EtOH (control)	8.56	100.0
RNase + C_6-AHB [incubation] + buffer + RNA	2.91	34.0
RNA + C_6-AHB [incubation] + buffer + RNase	8.40	98.1
Buffer + C_6-AHB [incubation] + RNA + RNase	8.45	98.7
RNA + buffer [incubation] + C_6-AHB + RNase	8.50	99.3

experiments were also carried out with chymotrypsin (EC 3.4.21.1) purchased from Merck (Germany).

RNase activity was assayed by the method of Anfinsen *et al.* [10] in a modification described in the handbook [9] using high-polymeric yeast RNA purchased from Sigma (United States). The amount of acid-soluble products formed from RNA at 37°C in 15 min was determined by measuring the optical density of the reaction mixture at λ = 260 nm (OD_{260}). The reaction mixture contained 0.1 ml of RNase solution (0.5 mg/ml), 0.1 ml of C_6-AHB solution in 5% ethanol (the blank reaction mixture contained 0.1 ml of 5% ethanol), 0.2 ml of 0.2 M Tris–HCl buffer (pH 8.5), and 0.1 ml of RNA solution (5 mg/ml). The reaction components were mixed in the aforementioned order, unless otherwise stated. In some experiments, the reaction was carried out at different pH values.

Chymotrypsin activity was determined from the rate of hydrolysis of *N*-benzoyl-L-tyrosine ethyl ester (BTEE) [11]. The reaction mixture contained 1.9 ml of BTEE solution (172 μg/ml) in Tris–HCl buffer (pH 7.8) with 0.1 M $CaCl_2$, 20 μl of enzyme solution (100 μg/ml) and 0.1 ml of C_6-AHB solution in ethanol (the blank reaction mixture contained 0.1 ml of ethanol). The reaction was carried out at 18–20°C for 15–75 s. Enzyme activity was calculated, using the coefficient of molar extinction equal to 900, from the absorbance of the products of BTEE hydrolysis measured at λ = 257 nm (SF-56 spectrophotometer; 10-mm cuvette).

The photooxidation of RNase was studied as described by Kurinenko *et al.* [12]. One and a half milliliters of RNase solution (0.5 mg/ml) in 0.1 M Tris–acetate buffer (pH 7.5) was mixed with 0.3 ml of C_6-AHB solutions to give the final concentrations of C_6-AHB equal to 0.01, 0.03, and 0.05%. After incubating the mixtures for 10, 30, and 60 min, they were mixed with 0.2 ml of a solution of methylene blue to give its final concentration of 0.0125%. The reaction mixtures containing the dye were placed on a magnetic stirrer (200–250 rpm) and exposed (25–28°C; 30 min)



Dependence of RNase activity on pH at different concentrations of C_6-AHB (%): (*1*) 0; (*2*) 0.03; (*3*) 0.05; (*4*) 0.08; and (*5*) 0.1.

Table 3. Effect of C_6-AHB on the photooxidation of RNase

C_6-AHB concentration, %	RNase activity	
	(U/mg protein) $\times 10^{-5}$	%
0 (before photooxidation)	8.57	100
0 (after photooxidation)	6.16	71.9
0.01	6.98	81.4
0.03	6.51	76.0
0.05	5.91	69.0

Table 4. Effect of the time of preincubation of 0.05% C_6-AHB with RNase on enzyme resistance to photooxidation

Preincubation time, min	RNase activity	
	(U/mg protein) $\times 10^{-5}$	%
Control 1 (before photooxidation)	8.56	100.0
Control 2 (after photooxidation without C_6-AHB)	6.16	72.0
0	5.91	69.0
10	6.60	77.1
30	7.54	88.1
60	8.34	97.4

to the light of a 200-W incandescent lamp placed at a distance of 22 cm. After exposure, the reaction mixtures were assayed, in 0.1-ml aliquots, for RNase activity.

The thermostability of RNase was determined as follows. Solutions containing RNase and C_6-AHB at a concentration of 0.05% were preincubated for 0, 10, 30, and 60 min and kept at 100°C for the next 20 min. In the control experiment (0-min preincubation), C_6-AHB was added to an RNase solution preheated to 100°C. After cooling the solutions to room temperature, RNase activity was assayed in 0.1-ml aliquots.

The thermostability of chymotrypsin was determined analogously, by incubating the enzyme at 60°C without (control) or with C_6-AHB at concentrations of 0.05 and 0.1%.

All the experiments were carried out in at least five replicates. The data obtained were statistically processed using Student's *t*-test with significance level $P < 0.05$.

RESULTS AND DISCUSSION

The stabilizing effect of the d_1 factor on the molecular structure of enzymes was estimated from its ability to prevent the inactivation of the enzymes exposed to a physical (heat, radiation) or chemical (pH value) damaging agent. In designing the experiments, we took into account that the stabilizing effect of the d_1 factor, which is presumably due to its binding to enzymes, must depend on the concentration of this factor and incubation time. Furthermore, like other d_1 factors of microorganisms, C_6-AHB is an amphiphilic compound, which occurs in aqueous media and in different compartments of cells either as a true solution (at concentrations less than 4.5×10^{-3} M or 0.09%) or a micellar colloid (at higher concentrations) [7].

Table 1 summarizes the results of experiments in which C_6-AHB was added to RNase solutions immediately before enzyme activity was measured. At pH 8.5 (optimum pH value for enzyme activity), the inhibitory effect of C_6-AHB directly depended on its concentration. At pH 7.0 and 6.0, the decrease in enzyme activity was obviously due to both nonoptimum pH values (this follows from the comparison of enzyme activities at a zero concentration of the d_1 factor) and the interaction of C_6-AHB with the enzyme. At concentrations close to the critical micelle concentration (CMC) value (0.09%), the inhibitory effect of C_6-AHB on RNase somewhat decreased: the remaining RNase activity at pH 6.0 and C_6-AHB concentrations of 0.08 and 0.1% was, respectively, 1.5-fold and 2-fold higher than at pH 8.5 (Table 1 and Fig. 1).

In the case of high-polymeric substrates, changes in the enzyme reaction rates may be due to modifications in the tertiary structure of not only enzymes but also substrates, as demonstrated by Filimonova *et al.* [13] with reference to the effect of Mg^{2+} ions on the rate of RNA hydrolysis by the *Serratia marcescens* nuclease. In our earlier work [7], we presented direct evidence that AHB inhibits RNase activity by complexing with the enzyme. In the present work, it was necessary to show that C_6-AHB did not interact with RNA under the experimental conditions chosen, although such interaction is basically possible [14].

With this in mind, we investigated the effect of a 15-min preincubation of C_6-AHB with different components of the reaction mixture on RNase activity (Table 2). The chosen concentration of C_6-AHB (0.05%) was sufficient to reliably inhibit the activity of RNase (see Table 1) and to cause a transition of microbial cells to the dormant state.

As can be seen from the data presented in Table 2, preincubation of RNase with C_6-AHB in the absence of RNA led to a considerable decrease in enzyme activity, whereas preincubation of C_6-AHB with RNA did not affect RNase activity. Therefore, in the experiments described in the present paper, the prevention of enzyme activity by C_6-AHB may be evidence for enzyme stabilization. It should be noted that the stabilizing effect of C_6-AHB depended on the time of its preincubation with the enzyme.

Resting microbial forms are subject to maturation; that is, cells require some time to attain the state of metabolic dormancy and resistance to hostile conditions. A similar phenomenon of the structural maturation of the enzyme–ABH complex was revealed in the following set of experiments on the photooxidation of RNase.

Table 5. Effect of the time of preincubation of 0.05% C_6-AHB with RNase on enzyme resistance to 20-min boiling

Preincubation time, min	RNase activity		
	(U/mg protein) $\times 10^{-5}$	% of control 1	% of control 2
Control 1 (before boiling)	8.56	100	–
Control 2 (after boiling without C_6-AHB)	8.23	96.1	100
0	5.35	62.5	65.0
10	5.43	63.4	66.0
30	6.09	71.1	74.0
60	6.91	80.7	84.0

Without preincubation, C_6-AHB, at concentrations of 0.01 and 0.03%, somewhat prevented (by about 10 and 4%, respectively) RNase photooxidation (Table 3). The absence of the protective effect of the higher concentration of C_6-AHB (0.05%) can be explained by the inhibitory action of this concentration of the d_1 factor on RNase (see Table 1).

Preincubation of RNase with 0.05% C_6-AHB led to enzyme stabilization (Table 4). The stabilizing effect increased with the time of preincubation, so that a 60-min preincubation of RNase with 0.05% C_6-AHB almost completely prevented the photooxidation of the enzyme. It is likely that preincubation stabilizes RNase by changing its conformation due to hydrophobic interactions and the formation of hydrogen bonds. Stabilized RNase becomes more resistant to denaturation processes, including photooxidation.

Since resting microbial forms exhibit increased thermoresistance, it would be reasonable to suggest that the interaction of C_6-AHB with RNase enhances its thermostability. This suggestion was verified using two enzymes differing in thermostability.

It is known that the high thermostability of *B. intermedius* RNase is due to the absence of disulfide bonds in its molecule [8]. As a result, cooling of the enzyme solution preliminarily boiled for 20 min (the so-called interval of cooperative melting) led to the restoration of the native enzyme structure [15]. As can be seen from the data presented in Table 5, the 20-min boiling of RNase did not noticeably affect their activity. When C_6-AHB was added to a hot RNase solution (0-min preincubation) or when it was preincubated with the enzyme for a short time period (10 min), enzyme renaturation was hindered, presumably due to the intercalation of C_6-AHB molecules into the enzyme molecule. In the case of a longer preincubation (30 or 60 min), the adverse effect of C_6-AHB on enzyme renaturation decreased, while its protective effect increased (Table 5).

Therefore, the interaction of C_6-AHB molecules with RNase is nonspecific; this follows from the equally efficient binding of the d_1 factor to both native and denatured forms of the enzyme. This inference agrees with our earlier findings showing the nonspecific interaction of AHBs with various protein macromolecules and explains the role of AHBs as microbial dormancy autoinducers [7].

Data on the interaction of C_6-AHB with chymotrypsin, which differs from RNase in being thermolabile, are summarized in Table 6. It can be seen that the heating of chymotrypsin at 60°C for 10 min in Tris–HCl buffer (pH 7.8) containing 1% $CaCl_2$ led to the complete inactivation of the enzyme. C_6-AHB at concentrations of 0.05 and 0.1% prevented the thermoinactivation of chymotrypsin: its residual activities at these concentrations of the d_1 factor were 95.6 and 62.5%, respectively. The difference in the protective effects of 0.05 and 0.1% C_6-AHB was probably due to different forms in which this d_1 factor exists in the medium at the indicated concentrations (true solution and micellar colloid, respectively).

Thus, by the mechanism of action (structural stabilization of protein globules), d_1 factors can be referred to natural chemical chaperons [16]. The interaction of AHBs with enzyme molecules raises their rigidity [17], due to which the activity of enzymes falls while their stability rises. The increased stability (in particular, thermostability) of proteins can be evidence for their structural modification [17, 18]. In our opinion, such a stabilizing effect of AHBs is convincingly demonstrated by the profound protective effect of C_6-AHB on RNase subjected to heat inactivation (Table 5, 60-min preincubation). The comparison of the results of 60-min and 0-min preincubations suggests that the prevention of enzyme activity in this case was not evidently due to protein refolding (Table 5).

Inasmuch as enzymes isolated from spores and vegetative cells are identical [19], it can be suggested that the aforementioned interactions of AHBs with protein macromolecules may be responsible for the suppression of metabolism and the development of the general resistance of resting microbial forms. Together with cell dehydration, the structural stabilization of enzymes, including nucleic acid depolymerases, by AHBs can explain cell survival in the absence of any energy-generating processes.

We believe that the data presented in this paper will provide further insight into the mechanisms of development and maintenance of dormant microbial forms, as

Table 6. Effect of C_6-AHB on chymotrypsin resistance to 15-min heating at 60°C

C_6-AHB concentration, %	Activity of chymotrypsin, mmol/s	
	before heating	after heating
0	0.88 (100 %)	0.0 (0 %)
0.05	0.69 (100 %)	0.66 (95.6 %)
0.1	0.56 (100 %)	0.35 (62.5 %)

well as into the regulatory functions of dormancy autoinducers in growing microbial cultures and associations at the population level. The possibility of the post-translational regulation of the catalytic activity and stability of enzymes should also be taken into account when considering the succession and function of microbial communities under varying environmental conditions.

Our interest in dormancy autoinducers is largely determined by the fact that they are always present in growing microbial cultures [1, 20–22]. The interactions of d_1 factors (AHBs) with enzymes and the dependence of these interactions on the protein-to-AHB molar ratios, AHB composition, and various environmental factors may represent a novel level of regulation of the physiological activity of microbial cultures and their functioning in situ.

ACKNOWLEDGMENTS

This work was supported by the Russian Foundation for Basic Research, projects nos. 97-04-49748 and 99-04-48639.

REFERENCES

1. Babusenko, E.S., El'-Registan, G.I., Gradova, N.B., Kozlova, A.N., and Osipov, G.A., Investigation of the Membranotropic Autoregulatory Factors of Methane-oxidizing Bacteria, *Usp. Khim.*, 1991, vol. 60, no. 11, pp. 2362–2373.
2. Mulyukin, A.L., Lusta, K.A., Gryaznova, M.N., Kozlova, A.N., Duzha, M.V., Duda, V.I., and El'-Registan, G.I., Generation of Resting Forms of *Bacillus cereus* and *Micrococcus luteus*, *Mikrobiologiya*, 1996, vol. 65, no. 6, pp. 782–789.
3. Osipov, G.A., El'-Registan, G.I., Svetlichnyi, V.A., Kozlova, A.N., Duda, V.I., Kaprel'yants, A.S., and Pomazanov, V.V., About the Chemical Nature of the Autoregulatory d Factor of *Pseudomonas carboxydoflava*, *Mikrobiologiya*, 1985, vol. 54, no. 2, pp. 184–190.
4. Batrakov, S.G., El'-Registan, G.I., Pridachina, N.N., Nenasheva, V.A., Kozlova, A.N., Gryaznova, M.N., and Zolotareva, I.N., Tyrosol, the Autoregulatory d_1 Factor of *Saccharomyces cerevisiae*, *Mikrobiologiya*, 1993, vol. 62, no. 4, pp. 633–638.
5. Vostroknutova, G.N., Kaprel'yants, A.S., Svetlichnyi, V.A., El'-Registan, G.I., Shevtsov, V.V., and Ostrovskii, D.N., Membranotropic Properties of the Autoregulatory Factor from a Bacterial Culture Liquid, *Prikl. Biokhim. Mikrobiol.*, 1983, vol. 19, no. 4, pp. 547–551.
6. Kaprel'yants, A.S., Suleimenov, M.K., Sorokina, A.D., Deborin, G.A., El'-Registan, G.I., Stoyanovich, F.M., Lille, Yu.E., and Ostrovskii, D.N., Structural and Functional Changes in Bacterial and Model Membranes Induced by Phenolic Lipids, *Biol. Membr.*, 1987, vol. 4, no. 3, pp. 254–261.
7. Bespalov, M.M., Kolpakov, A.I., Loiko, N.G., Doroshenko, E.V., Mulyukin, A.L., Kozlova, A.N., Varlamova, E.A., Kurganov, B.I., and El'-Registan, G.I., The Role of Microbial Dormancy Autoinducers in Metabolism Blocking, *Mikrobiologiya*, 2000, vol. 69, no. 2, pp. 217–223.
8. Golubenko, I.A., Balaban, N.P., Leshchinskaya, I.B., Volkova, T.I., Kleiner, G.I., Chepurnova, N.K., Afanasenko, G.A., and Dudkin, S.M., Ribonuclease of *Bacillus intermedius* 7R: Purification by Chromatography on Phosphocellulose and Some Characteristics of the Homogenous Enzyme, *Biokhimiya*, 1979, vol. 44, pp. 640–648.
9. Leshchinskaya, I.B., Balaban, N.P., Kapranova, M.N., and Golubenko, I.A., Assay Methods for Nucleases and Related Enzymes, *Sovremennye metody izucheniya nukleinovykh kislot i nukleaz mikroorganizmov* (Modern Methods of Investigation of Microbial Nucleic Acids and Nucleases), Kazan: Kazan State Univ., 1980, pp. 53–57.
10. Anfinsen, C.B., Reofield, R.P., Choate, W.L., *et al.*, Studies of the Gross Structure, Cross-Linkages, and Terminal Sequences in Ribonuclease, *J. Biol. Chem.*, 1954, vol. 207, pp. 201–210.
11. Aisina, R.B., Kazanskaya, N.F., Lukasheva, E.V., and Berezin, I.V., Production and Properties of Microencapsulated α-Chymotrypsinogen, *Biokhimiya*, 1976, vol. 41, no. 9, pp. 1656–1661.
12. Kurinenko, B.M., Golubenko, I.A., Bulgakova, R.Sh., Davydov, R.E., Nekhoroshkova, Z.M., Valiullina, S.F., and Shlyapnikov, S.V., Photooxidation of the *Bacillus intermedius* 7P Ribonuclease and Isolation of the Photoinactivated Enzyme, *Bioorg. Khim.*, 1986, vol. 12, no. 4, pp. 457–466.
13. Filimonova, M.N., Gubskaya, V.P., Nuretdinov, I.A., Benedik, M.J., Bogomol'naya, L.M., Andreeva, M.A., and Leshchinskaya, I.B., *Serratia marcescens* Nuclease Isoenzymes: Role of Mg^{2+} Cations in the Mechanism of Hydrolysis, *Biokhimiya*, 1997, vol. 62, no. 9, pp. 1148–1154.
14. Lytollis, W., Scannel, R.T., An, H., Murty, V.S., Reddy, K.S., Barr, J.R., and Hecht, S.M., 5-Alkylresorcinols from *Hakea trifurcata* that Cleave DNA, *J. Am. Chem. Soc.*, 1995, vol. 117, pp. 12 683–12 690.
15. Faizullin, D.A. and Stupishina, E.A., Effect of Conformational Factors on the Reversibility of Heat Denaturation of Binase, *Fermenty mikroorganizmov* (Microbial Enzymes), Kazan, 1998, pp. 109–115.
16. Tatzelt, J., Prusiner, S.B., and Welch, W.J., Chemical Chaperones Interfere with the Formation of Scrapie Prion Protein, *EMBO J.*, 1996, vol. 15, no. 23, pp. 6363–6373.

17. Margolin, A.L., Sherstyuk, S.F, Izumrudov, V.A., Shvyadas, V.Yu., Zezin, A.B., and Kabanov, V.A., Phase Transitions in Polyelectrolyte Solutions as a Model for Spore Formation, *Dokl. Akad. Nauk SSSR*, 1983, vol. 272, no. 1, pp. 230–233.

18. Martinek, K., *Uspekhi bioorganicheskogo kataliza* (Advances in Bioorganic Catalysis), Moscow: Mosk. Gos. Univ., 1979, pp. 105–157.

19. Ferano, H., Fujita, Y., and Hiroishi, S., *Agr. Biol. Chem*, 1975, vol. 39, pp. 2057–2063.

20. Svetlichnyi, V.A., Romanova, A.K., and El'-Registan, G.I., Concentration of Membranotropic Autoregulators in Lithoautotrophically Grown *Pseudomonas carboxydoflava, Mikrobiologiya*, 1986, vol. 55, no. 1, pp. 55–59.

21. El'-Registan, G.I., Duda, V.I., Svetlichnyi, V.A., Kozlova, A.N., and Tipiseva, I.A., Dynamics of Autoregulatory d Factors in Batch Cultures of *Pseudomonas carboxydoflava* and *Bacillus cereus, Mikrobiologiya,* 1983, vol. 52, no. 2, pp. 238–243.

22. Sharipova, M.R., Filatova, S.V., Vinter, V.G., and Leshchinskaya, I.B., Investigation of the Intracellular and Extracellular Ribonucleases of *Bacillus intermedius, Mikrobiologiya*, 1984, vol. 53, no. 4, pp. 563–567.