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Intensity of Free Radical Processes and Regulation of Cytoplasmic NADP-Isocitrate Dehydrogenase in Rat Cardiomyocytes under Normal and Ischemic Conditions

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Abstract—The intensity of free radical processes and the regulation of NADP-isocitrate dehydrogenase (EC 1.1.1.42; NADP-IDH) activity have been studied in the cytoplasmic fraction of normal and ischemized rat myocardium. Chemiluminescence parameters, such as the light sum (S) of slow flash and the tangent of the kinetic curve slope angle ($\tan\alpha_1$), which characterize the intensity of free radical processes, were increased in ischemia 2.1- and 20.0-fold, respectively. The slow flash intensity (I_{max}) was increased 22-fold. The contents of lipid peroxidation products—diene conjugates and malonic dialdehyde—were increased 11.9- and 4.7-fold, respectively, suggesting pronounced oxidative stress. Using homogenous enzyme preparations of NADP-IDH isolated from the normal and experimentally ischemized rat myocardium, a number of catalytic properties of the enzyme were characterized for normal and pathologic conditions. NADP-IDH from the normal and ischemized myocardium had the same electrophoretic mobility and was regulated similarly by Fe^{2+}, Cu^{2+}, Zn^{2+}, and also with succinate and fumarate. However, under normal and pathologic conditions NADP-IDH was different in the affinity for substrates and in the sensitivity to inhibitory effects of hydrogen peroxide, reduced glutathione, and of Ca^{2+}. The degree of synergy in the enzyme inhibition with Fe^{2+} and H_2O_2 was less pronounced in ischemia. The inhibitory effect of the reaction product 2-oxoglutarate was higher under normal conditions than in ischemia (the K_i values were 0.22 and 0.75 mM, respectively). The specific features of the NADP-IDH regulation in ischemia are suggested to promote the stimulation of the enzyme functioning during increased level of free radical processes, and this seems to be important for NADPH supplying for the glutathione reductase/glutathione peroxidase antioxidant system of cardiomyocytes.

Key words: rat heart, normal and ischemic conditions, free radical processes, NADP-isocitrate dehydrogenase, properties, activity regulation

Free radical processes play an important role in the development of ischemic heart disease and myocardial infarction [1, 2]. Available data suggest that reactive oxygen species (ROS) generated during mitochondrial and microsomal oxidation as a result of an incomplete oxygen reduction to water should be immediately involved in the development of myocardial tissue damage [3-5]. Free radical processes are controlled by a multilevel system of the protection that includes enzymatic and nonenzymatic mechanisms responsible for maintenance of a certain level of ROS [5, 6]. Functions of this system on the subcellular level are provided by a number of processes coupled, in particular, with changes in contents of respiratory substrates [7, 8], some metal ions, especially Fe^{2+} [5, 6, 9, 10], Ca^{2+} [8, 10, 11], and Cu^{2+} [10, 12], in the level of NADP/NADPH [13], etc.

The enzyme system of glutathione reductase/glutathione peroxidase, which is responsible for detoxification of H_2O_2, plays an important role in antioxidant protection. This is especially important for elimination of the most aggressive ROS form, a hydroxyl radical $OH^{\cdot}$ generated from H_2O_2 in Fenton's reaction [5, 6]. The activity of

Abbreviations: NADP-IDH) NADP-dependent isocitrate dehydrogenase; ROS) reactive oxygen species.

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the glutathione reductase/glutathione peroxidase system is determined by the NADPH level in the cell that supports the reduced state of glutathione [10].

There is a suggestion that in the animal heart muscle NADPH is generated not only through the pentose phosphate pathway, but mainly through the reaction catalyzed by NADP-dependent isocitrate dehydrogenase (EC 1.1.1.42; NADP-IDH) [14]. The generation of NADPH during the NADP-IDH reaction is connected with oxidative decarboxylation of isocitrate to 2-oxoglutarate. There are data on specific features of the functioning of NADP-IDH in a number of plant and animal tissues, including data for the enzyme isolated from heart [15-18]. The functioning of this enzyme is thought to be connected with biosyntheses in the cell. The involvement of NADP-IDH in the coordination of carbon and nitrogen metabolism is a subject of recent intensive studies and discussions because the product of this reaction, 2-oxoglutarate, seems to be a precursor of the most important amino acids, glutamate and glutamine [18]. However, it should be taken into account that NADP-IDH also provides the production of NADPH, which seems to be a significant alternative source of reducing equivalents contributing the NADPH generation through the pentose phosphate pathway. But this problem remains virtually unstudied.

The abovementioned aspect of the enzyme action can be directly associated with the realization of antioxidant protection of the organism under conditions of ischemic damage to the myocardium by the enzymatic system glutathione reductase/glutathione peroxidase; therefore, the purpose of the present work was to study and comparatively characterize kinetic properties and the regulation of activity of NADP-IDH from rat heart under normal and ischemic conditions.

MATERIALS AND METHODS

Male white rats with body weight of 250-300 g fed with a standard diet were used. The myocardium was ischemized by 45-min occlusion of the descending branch of the left coronary artery at the level of the left *auricula atrii* [7, 19].

The intensity of free radical oxidation in the myocardium was assessed by the Fe^{2+}-induced chemiluminescence in the cytoplasm of cardiomyocytes [20] and also by spectrophotometric determination of the primary and secondary products of lipid peroxidation: diene conjugates [21] and malonic dialdehyde [22]. Differential absorption spectra of cytoplasmic lipids extracted into the heptane phase were also recorded [8].

The activity of NADP-IDH was determined spectrophotometrically. The rate of the enzymatic reaction was assessed by the increase in the optical density at 340 nm as a result of NADP reduction during oxidative decarboxylation of isocitrate. The activity of NADP-IDH was determined in spectrophotometry medium of the following composition: 50 mM Tris-HCl buffer (pH 7.6) containing 0.1 mM $MnCl_2$, 0.05 mM isocitrate, 0.4 mM NADP. The enzyme amount catalyzing the generation of 1 μmol product for 1 min at 25°C was taken as the activity unit. The total protein was determined by the Lowry method [23].

The cell fractions were separated and the subcellular location of NADP-IDH was studied by differential centrifugation [24]. A sample of the heart muscle was homogenized in a cooled ceramic mortar with a threefold volume of the isolation medium as follows: 50 mM Tris-HCl buffer (pH 7.6), 10 mM EDTA, 0.3 M sucrose, 0.5 mM β-mercaptoethanol. The homogenate was filtered through a nylon layer with square holes (0.1 mm) and centrifuged at 3500*g* for 10 min to remove nondestroyed tissue elements and cardiomyocyte membranes. The supernatant was centrifuged at 15,000*g* for 20 min. The precipitate which mainly contained mitochondria was resuspended with 50 mM Tris-HCl buffer (pH 7.6) containing 0.175 M KCl and centrifuged again at 15,000*g* for 15 min. NADP-IDH was solubilized in a Potter's homogenizer with 50 mM Tris-HCl buffer (pH 7.6), 0.1 mM EDTA, 0.5 mM β-mercaptoethanol, 20% glycerol, and 1% Triton X-100. The activities of NADP-IDH and of marker enzymes, such as succinate dehydrogenase (a mitochondrial marker) [25] and lactate dehydrogenase (a cytosolic marker) [26], were determined in the supernatant which was a cytoplasmic fraction of cardiomyocytes and in the resulting mitochondrial precipitate.

The cytoplasmic NADP-IDH from the myocardium of normal and ischemized animals was purified by several stages. The myocardium cytoplasmic fraction isolated by differential centrifugation was fractionated with ammonium sulfate, and the protein fraction precipitated at the 30-60% saturation of $(NH_4)_2SO_4$ was isolated. The resulting precipitate was resuspended with 1 ml of the isolation medium. The enzyme solution free of low-molecular-weight admixtures was prepared by filtration on a column (1.4 × 20 cm) with Sephadex G-25 (fine). NADP-IDH was eluted with 10 mM Tris-HCl buffer (pH 7.8) containing 0.5 mM β-mercaptoethanol and 0.1 mM EDTA. Then NADP-IDH was purified by ion-exchange chromatography on a column (0.75 × 13 cm) with DEAE-cellulose. The enzyme was desorbed by a stepwise increase in the concentration of KCl up to 80 mM. Then gel chromatography on Sephadex G-150 (45 × 2 cm) was performed. The enzyme isolation and purification stages were performed in a cold chamber at 0-4°C.

Electrophoresis in 7.5% polyacrylamide gel was performed by the method of Davis [27]. The protein was revealed by staining with Coomassie Blue R-250.

The resulting enzyme preparations were used to compare the catalytic properties of NADP-IDH and the regulation of its activity under normal and ischemic conditions. The inhibition type and constants were determined as described in [28].

The experiments were performed in three biological replicates. Analytical determinations for each sample were performed in two replicates. The reliability of results was controlled by methods of variation statistics [29]. The plots were drawn using programs of linear and parabolic approximation.

The following reagents and materials were used: Sephadex G-25 and Sephadex G-150 (Pharmacy, Sweden); DEAE-cellulose (Whatman, Great Britain); Tris (Serva, Germany); isocitrate, 2-oxoglutarate (Sigma, USA); NADP, reduced glutathione (Reanal, Hungary). Other reagents of chemical or analytical purity were of domestic production.

Table 1. Contents of lipid peroxidation products in the cytoplasm of rat cardiomyocytes under normal and experimental ischemic conditions of the myocardium

Experimental conditions	Diene conjugates, μM	Malonic dialdehyde, μM	α-Tocopherol, μM
Norm	0.24 ± 0.03	0.96 ± 0.10	2.30 ± 0.90
Ischemia	2.86 ± 0.05	4.52 ± 0.30	10.50 ± 1.30

RESULTS AND DISCUSSION

Tables 1 and 2 present the contents of products of lipid peroxidation and also the intensity parameters of chemiluminescence signal in the cytoplasmic fraction of rat cardiomyocytes under normal and experimental ischemic conditions. In the ischemized rat myocardium the levels of diene conjugates and of malonic dialdehyde were increased 11.9- and 4.7-fold, respectively, this suggesting pronounced oxidative stress. The differential absorption spectra of the cytoplasmic lipids extracted into the heptane phase displayed the absorption maximum at 233 nm specific for hydroperoxides. The parameters of chemiluminescence intensity, such as the light sum (S) of slow flash and the tangent of the kinetic curve slope angle ($\tan\alpha_1$), which characterize the intensity of free radical processes, were increased in ischemia 2.1- and 20.0-fold, respectively. The slow flash intensity (I_{max}) in the cytoplasm of cardiomyocytes from rats with ischemic damage of the myocardium was increased 22.0-fold compared to normal. The I_{max} value on Fe-induced chemiluminescence is proportional to the concentration of lipid hydroperoxides [30]. However, under conditions of

Table 2. Chemiluminescence parameters of the cytoplasmic fraction of rat cardiomyocytes under normal and experimental ischemic conditions of the myocardium

Experimental conditions	Light sum of the slow flash, S	Tangent of the angle of the increase in the slow flash, $\tan\alpha_1$	Tangent of the angle of the decrease in the chemiluminescence, $\tan\alpha_2$	Amplitude of the maximum flash, I_{max}
Norm	2.62 ± 0.20	0.24 ± 0.10	−0.08 ± 0.04	0.15 ± 0.05
Ischemia	5.40 ± 0.50	4.82 ± 0.16	−1.30 ± 0.09	3.37 ± 0.07

Table 3. Purification of the cytoplasmic NADP-isocitrate dehydrogenase from the normal and experimentally ischemized rat myocardium

Stage of purification	Experimental conditions	Volume, ml	Amount of protein, mg	Specific activity, units/mg protein	Yield, %	Degree of purification
Cytoplasmic fraction	norm	8.0	45.0 ± 1.38	0.17 ± 0.02	100.00	1.00
	ischemia	8.0	45.0 ± 1.40	0.31 ± 0.03	100.00	1.00
Fractionation with $(NH_4)_2SO_4$	norm	1.2	4.64 ± 0.25	0.83 ± 0.03	50.80	4.88
	ischemia	1.2	4.91 ± 0.27	1.41 ± 0.03	49.68	4.55
Gel filtration on Sephadex G-25	norm	9.0	4.60 ± 0.23	0.83 ± 0.02	50.66	4.88
	ischemia	9.0	4.73 ± 0.25	1.43 ± 0.02	48.53	4.61
Ion-exchange chromatography on CM-cellulose	norm	15.0	0.31 ± 0.05	6.71 ± 0.02	27.51	39.50
	ischemia	15.0	0.53 ± 0.05	8.02 ± 0.04	30.51	25.87
Gel chromatography on Sephadex G-150	norm	6.0	0.04 ± 0.03	19.00 ± 0.05	10.05	111.76
	ischemia	6.0	0.06 ± 0.03	32.50 ± 0.05	14.00	104.84

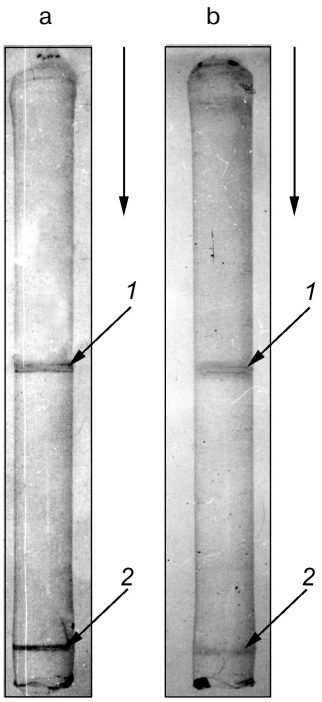

Fig. 1. Electrophoregrams of NADP-isocitrate dehydrogenase preparations from normal (a) and experimentally ischemized (b) rat myocardium: *1*) NADP-isocitrate dehydrogenase; *2*) the marker zone (Bromophenol Blue). The arrow shows the direction of the protein movement.

ischemia, the tanα_2 value which characterized the antioxidative activity of studied specimens was significantly higher than the corresponding parameter under normal conditions (Table 2), and this suggested the mobilization of the antioxidant system of cardiomyocytes in ischemia.

As to the subcellular location of NADP-IDH in the rat cardiomyocytes, the enzyme level was the highest in the cytoplasm (~85%) in both normal and ischemized myocardium. The mitochondrial fraction contained no more than ~15% of the total activity. Data on the activities of the marker enzymes of mitochondria and cytoplasm indicated that the cross contamination of the subcellular fractions was not higher than the parameters specific for the method used [24].

Under conditions of ischemia, the activities of NADP-IDH were increased 1.8- and 2.5-fold in the car-

Table 4. Values of K_m for the cytoplasmic NADP-IDH from the normal and ischemized rat myocardium

Experimental conditions	Values of K_m for substrates and cofactors, mM		
	isocitrate	Mn^{2+}	NADP
Norm	0.038 ± 0.002	0.040 ± 0.005	0.210 ± 0.004
Ischemia	0.012 ± 0.002	0.004 ± 0.001	0.140 ± 0.006

Table 5. The interaction of effects of some inhibitors on NADP-isocitrate dehydrogenase from normal and ischemized rat heart

Name of inhibitor	Concentrations of Fe^{2+}, mM	Normal		Ischemia		Type of inhibitor interaction
		A	B	A	B	
Fe^{2+} + hydrogen peroxide	0.02	1.00	0.92	0.95	0.94	
	0.04	0.80	0.63	0.82	0.71	
	0.08	0.49	0.33	0.59	0.54	synergism (A > B)
	0.12	0.23	0.20	0.31	0.33	
	0.16	0.17	0.15	0.24	0.27	
	0.20	0.13	0.14	0.18	0.20	
Fe^{2+} + glutathione	0.02	0.74	0.74	0.94	0.95	
	0.04	0.63	0.61	0.84	0.85	
	0.08	0.43	0.43	0.67	0.67	additive inhibition
	0.12	0.22	0.23	0.35	0.36	(A = B)
	0.16	0.17	0.17	0.27	0.28	
	0.20	0.13	0.13	0.22	0.22	

Note: A ($V_1/V_0 \times V_2/V_0$) is the product of relative rates of the enzymatic reaction in the presence of Fe^{2+} (V_1) and of the second inhibitor (V_2) and in the absence of the inhibitors (V_0); B ($V_{1,2}/V_0$) is the ratio of the enzymatic reaction rate in the presence and in the absence of the inhibitor pair.

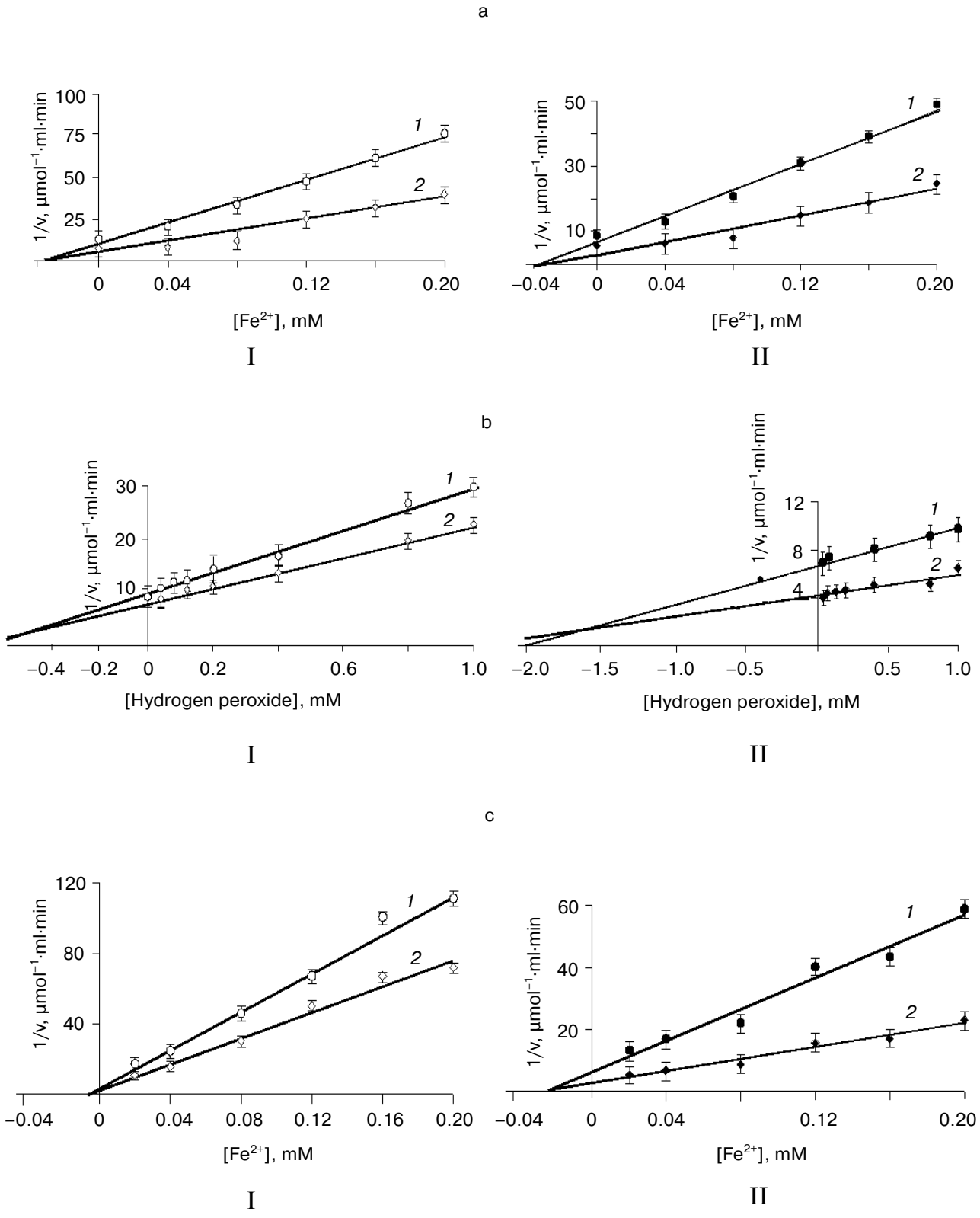

Fig. 2. Determination of the type and constants of inhibition of NADP-isocitrate dehydrogenase from the normal (I) and ischemized (II) rat myocardium at fixed concentrations of isocitrate with Fe^{2+} (a), H_2O_2 (b), and with Fe^{2+} in the presence of 0.10 mM H_2O_2 (c). Isocitrate concentrations: *1*) 0.01 mM; *2*) 0.05 mM.

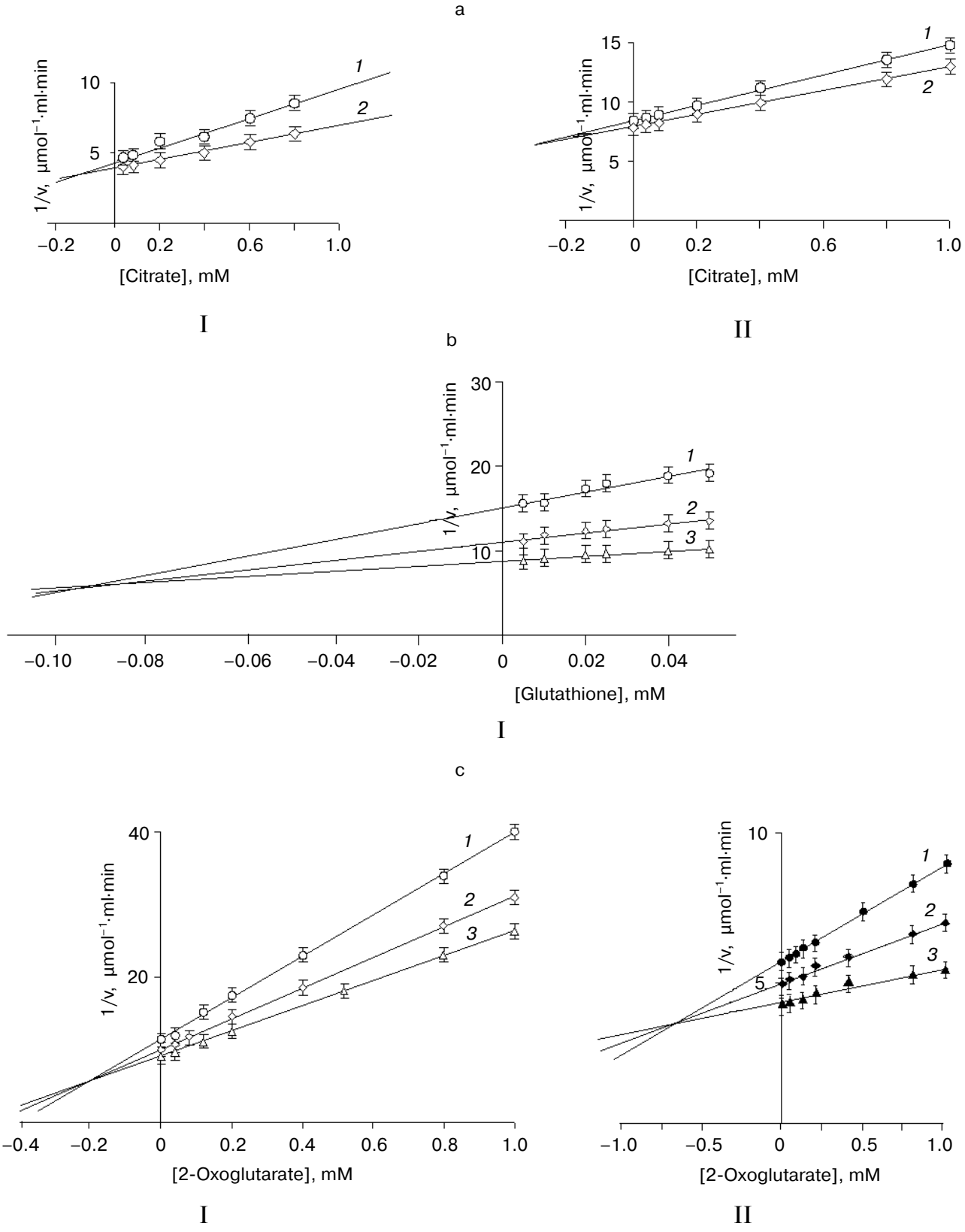

Fig. 3. Determination of the type and constants of inhibition of NADP-isocitrate dehydrogenase from the normal (I) and ischemized (II) rat myocardium at fixed concentrations of isocitrate with citrate (a), glutathione (b), and 2-oxoglutarate (c). Isocitrate concentrations: *1*) 0.01 mM; *2*) 0.05 mM; *3*) 0.10 mM.

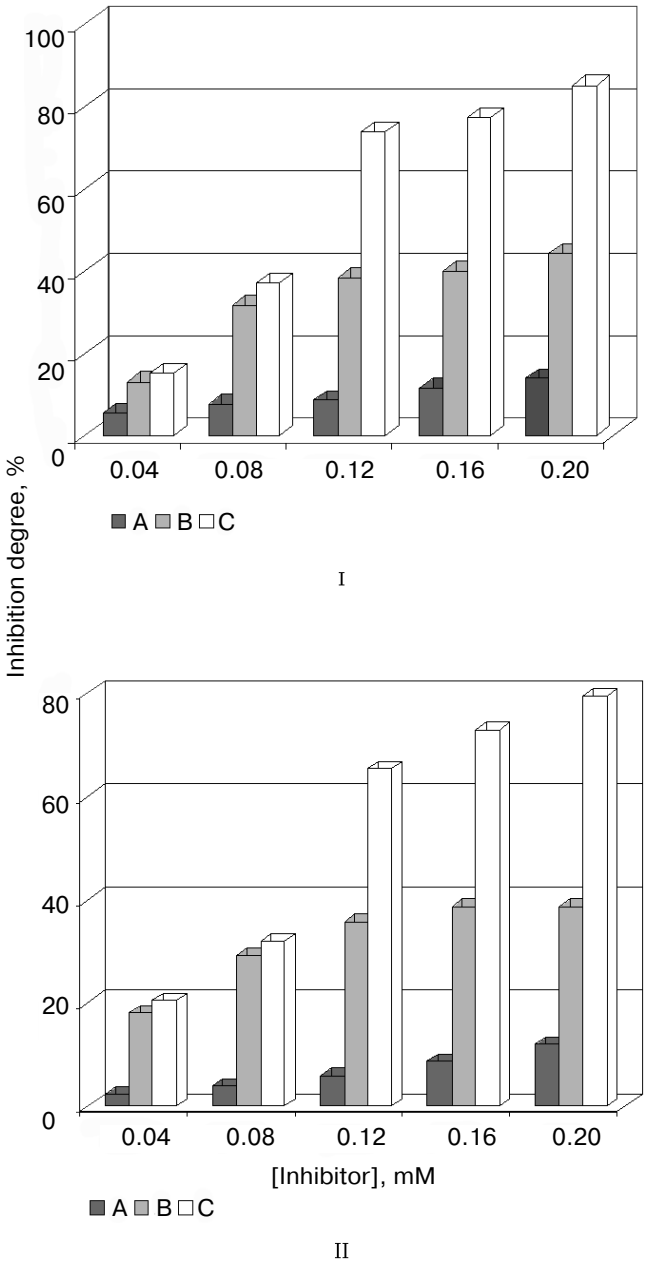

Fig. 4. Degree of inhibition of NADP-IDH from normal (I) and ischemized (II) rat myocardium at varied concentrations of Fe^{2+} (A), citrate (C), and of Fe^{2+} in the presence of citrate (B).

diomyocyte cytoplasm and mitochondria, respectively. Note, that under conditions of hypoxia and ischemia the activities of NADP-IDH in the cytoplasm of rat brain cortex cells were increased 26 and 43% and in the cerebellum 6 and 20%, respectively [31]. The interrelation between the damaging effect of ischemia and the increase in the IDH activity was also found in the cytoplasm of the rabbit spinal marrow [32]. The changes in the enzyme activity found in the cardiomyocytes suggested that this effect should occur under conditions of ischemic damage of various mammalian tissues. However, studies on the effect of ischemia on NADP-IDH are scarce and are limited to determination of the enzyme activity in disease. There are virtually no studies on the regulation of the NADP-IDH activity under conditions of ischemia. There is only the above-mentioned study on the NADP-IDH activity in the rat brain cortex and cerebellum, and the enzyme activities were found to decrease on addition of oleic and palmitic acids proportionally to their concentrations [31].

In the present work the 111.8- and 104.8-fold purification resulted in preparations of the cytoplasmic NADP-IDH from the normal and ischemized myocardium with the specific activities of 19.0 and 32.5 units per mg protein, respectively (Table 3). Electrophoretic studies confirmed the homogeneity of NADP-IDH in the maximally active fractions after gel chromatography on Sephadex G-150 (Fig. 1). The staining for protein in polyacrylamide gel showed the only band with the electrophoretic mobility $R_f = 0.51$.

The obtaining of highly purified NADP-IDH preparations allowed us to study a number of catalytic properties and the regulation of the enzyme activity in the normal and ischemized myocardium.

Values of Michaelis constants (K_m) for substrates of NADP-IDH from the normal and ischemized rat myocardium are presented in Table 4. Under conditions of ischemia, the enzyme affinity was shown to increase for isocitrate, $NADP^+$, and Mn^{2+}, which are essential cofactors of NADP-IDH.

The hydroxyl radical $OH^{\cdot}$ generated during Fenton's reaction is the most important contributor to the development of stress-induced tissue damage in ischemia [5, 6]. Therefore, the effect of the Fenton's reaction components on the NADP-IDH activity was studied.

It was found that Fe^{2+} displayed a noncompetitive inhibitory effect on the activity of NADP-IDH from both normal and ischemized rat myocardium (K_i values were 0.034 and 0.037 mM, respectively) (Fig. 2a). Hydrogen peroxide displayed a mixed type inhibition, with K_i values of 0.450 and 1.640 mM for the normal and ischemized myocardium, respectively (Fig. 2b). Note, that in the presence of hydrogen peroxide the K_i value of NADP-IDH from the normal myocardium for Fe^{2+} was significantly decreased (to 0.006 mM) that suggested a synergism in the effects of the inhibitors (Fig. 2c (I)). In ischemia the K_i value for Fe^{2+} in the presence of H_2O_2 was increased more than 3.3-fold compared to the normal value (K_i = 0.020 mM) (Fig. 2c (II)). Obviously, under conditions of ischemia the synergism degree of the effects of inhibitors under study was decreased. Data presented in Table 5 also confirmed the possibility of synergism of the inhibitory effects of Fe^{2+} and hydrogen peroxide on

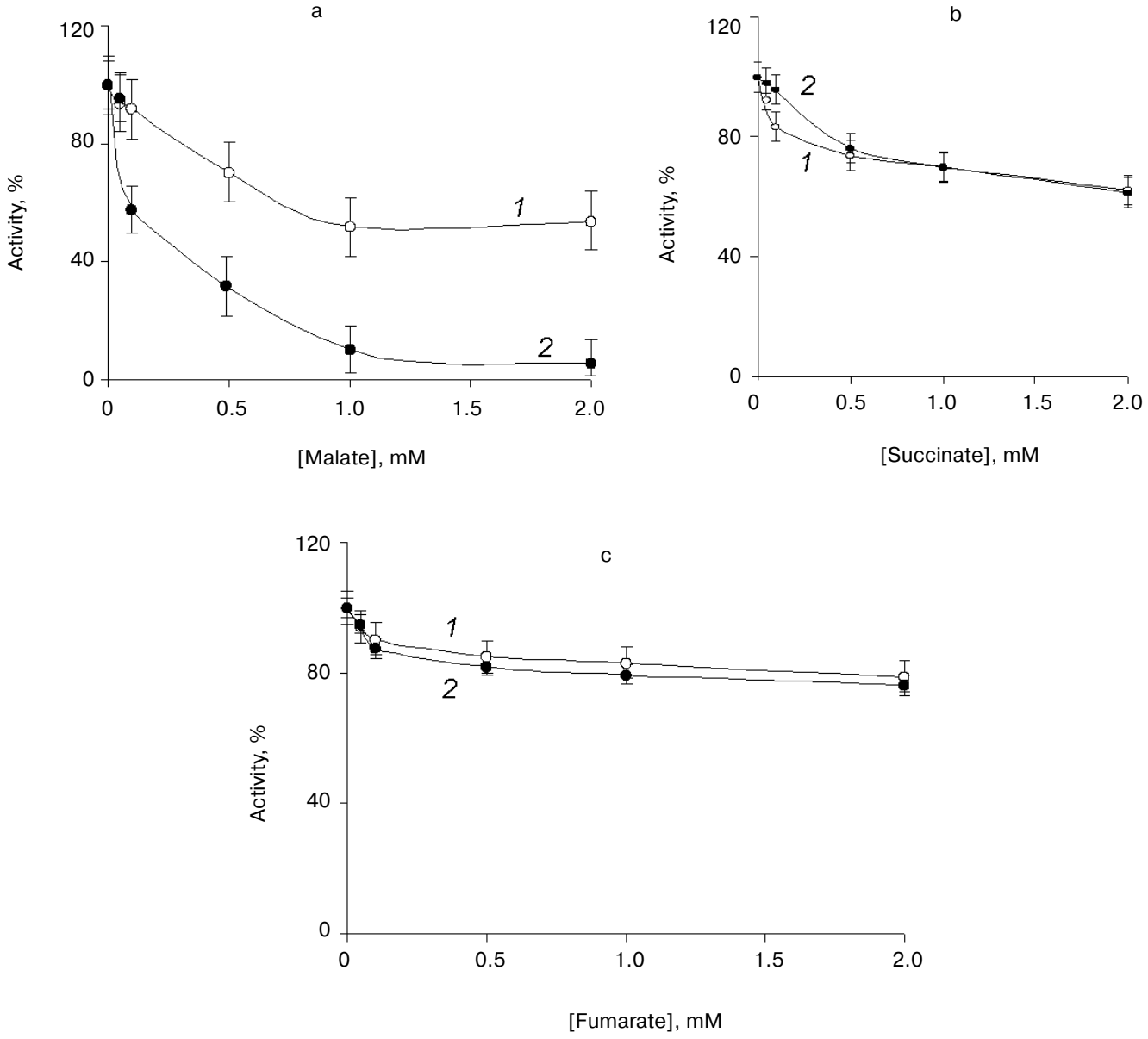

Fig. 5. Effects of malate (a), succinate (b), and fumarate (c) on the activity of NADP-isocitrate dehydrogenase from the normal (*1*) and ischemized (*2*) rat myocardium.

NADP-IDH and of a decrease in the degree of this synergism under conditions of ischemia.

Because citrate can markedly affect Fenton's reaction as an effective chelator of Fe^{2+} [6], the effect of this metabolite on the NADP-IDH activity was also studied. Citrate was a competitive inhibitor of the enzyme with K_i values of 0.215 and 0.125 mM under normal and ischemic conditions, respectively (Fig. 3a). The inhibitory effect of Fe^{2+} on NADP-IDH was decreased in the presence of citrate, which seems to be associated with a decrease in the level of free Fe^{2+} as a result of its binding to citrate (Fig. 4).

Studies on the effect of reduced glutathione, which acts as a reduced cofactor in the glutathione peroxidase reaction responsible for detoxification of H_2O_2, revealed a difference in its effects on NADP-IDH under normal and ischemic conditions. Thus, glutathione inhibited the enzyme from the normal myocardium; the inhibition was of the mixed type with $K_i = 0.087$ mM (Fig. 3b). But this metabolite had no effect in ischemia. The inhibitory

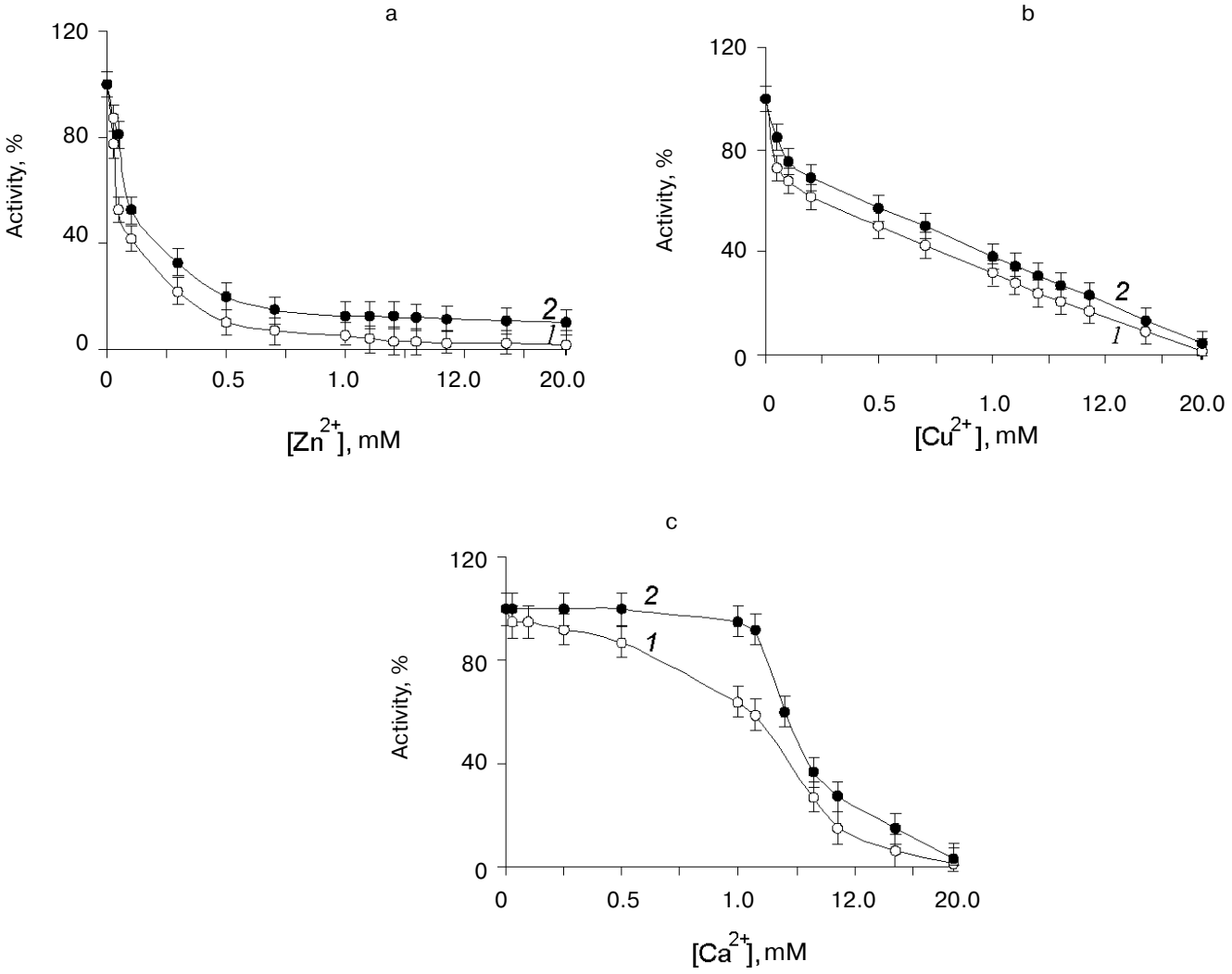

Fig. 6. Effects of metal ions on the activity of NADP-isocitrate dehydrogenase from the normal (*1*) and ischemized (*2*) rat myocardium: Zn^{2+} (a); Cu^{2+} (b); Ca^{2+} (c).

effects of Fe^{2+} and of reduced glutathione on the enzyme from the normal myocardium were additive (Table 5).

Effects of some dicarboxylic acids on the enzyme activity were studied, and the inhibition of NADP-IDH from the normal heart by 2-oxoglutarate, which is a product of the enzymatic reaction, was stronger. Note that the K_i values for NADP-IDH inhibition with 2-oxoglutarate were significantly different under normal and ischemic conditions (0.220 and 0.750 mM, respectively, Fig. 3c). Note also that occlusion of the left coronary artery in experimental myocardial infarction in dogs resulted in disorders in oxidation of a number of respiration substrates in mitochondria that was the most pronounced in the case of 2-oxoglutarate [33]. On taking into account that the mitochondrial membrane contains a carrier specific for 2-oxoglutarate [34-36], it was suggested that changes in the NADP-IDH sensitivity to the regulatory effect of this metabolite in ischemia should have special importance. On the contrary, malate displayed the stronger inhibitory effect on the enzyme isolated from the ischemized myocardium. The degrees of inhibition of the enzymatic activity with succinate and fumarate were virtually the same under normal and ischemic conditions (Fig. 5, a-c).

Effects of Ca^{2+}, Zn^{2+}, and Cu^{2+} on the NADP-IDH activity have also been studied because these ions are known to play a significant role in free radical oxidation and in metabolic changes in ischemia [8, 10-12, 37]. The inhibitory effects of Zn^{2+} and Cu^{2+} on NADP-IDH were similar under normal and ischemic conditions (Fig. 6, a and b). But the inhibitory effect of Ca^{2+} on NADP-IDH from the normal rat heart muscle was found at the concentration of 0.25 mM, whereas the enzyme from the ischemized myocardium was inhibited at the higher concentration of 1.20 mM (Fig. 6c).

Thus, the findings have indicated the presence of significant changes in the regulation of activity of the cytoplasmic NADP-IDH from rat myocardium under conditions of ischemia. The increased affinity of the enzyme for substrates and cofactors, the decreased suppression of the NADP-IDH activity with H_2O_2 and 2-oxoglutarate, and also the decreased synergism of the inhibitory effects of Fe^{2+} and H_2O_2 seem to promote intensification of the enzyme functioning under conditions of increased level of free radical processes, and this can be important for provision with NADPH of the glutathione reductase/glutathione peroxidase system of cardiomyocytes.

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