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Determination of Arachidonic Acid Based on the Prostaglandin H Synthase Catalyzed Reaction

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Abstract

This article presents a novel method of arachidonic acid (AA) determination based on the reaction catalyzed by prostaglandin H synthase (PGHs). The deoxygenated and nondeoxygenated (as control) buffers were used to obtain the PGHs preparations from bovine vesicular glands by two different methods. The higher specific activity was observed for solubilized preparations obtained by ultracentrifugation and deoxygenated buffers. The preparations obtained by Ca^{2+} treatment demonstrated higher stability of PGHs during its storage at –15°C. To record the initial rate of AA transformation, a spectrophotometric assay of PGHs cyclo-oxygenase and peroxidase activities was developed using adrenaline and ABTS as electron donors. No oxidation of ABTS was observed in the reaction of AA transformation catalyzed by the PGHs from bovine vesicular glands. However, this electron donor was successfully used in the reaction catalyzed by PGHs from sheep vesicular glands. No chemiluminescence was recorded in the reaction of AA transformation catalyzed by PGHs from bovine vesicular glands in the presence of luminol. The chemiluminescent intensity was measured after addition of hydrogen peroxide allowing quantitative assay of AA to be performed.

Index Entries: Prostaglandin H synthase; arachidonic acid; adrenaline; ABTS; luminol.

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Introduction

Determination of arachidonic acid (AA), as well as other polyunsaturated fatty acids (PUFA, precursors of prostanoids), is of great importance for diagnostics of some pathologies *(1)*. The specific analytical procedure commonly used to determine AA requires expensive manipulation and separation techniques *(1)*. We suppose that a spectrophotometric assay based on a specific reaction catalyzed by the prostaglandin H synthase (PGHs) might be proposed as an alternative. It will help to broaden determination of AA and PUFA in clinical and industrial laboratories.

Prostaglandin H synthase (EC 1.14.99.1) is the first and rate-limiting enzyme in the transformation of polyunsaturated fatty acids into prostanoids *(2–4)*. The enzyme exhibits two enzymatic activities (cyclo-oxygenase and peroxidase) and requires participation of heme and four molecules of substrates: polyunsaturated fatty acid, two oxygen molecules, and an electron donor. Adrenaline, NADH, hydroxyquinone, ferrocyanide, and homovanillic acid were studied as electron donors in the reaction catalyzed by PGHs from sheep vesicular glands *(5)*. This enzyme is undoubtedly of biotechnological interest, because it is used in the biosynthetic production of a wide array of prostaglandins *(2,6)*. These substances are in great demand as research reagents and in medicine *(7)*. Moreover, PGHs is therapeutically important because it is selectively inhibited by aspirin and related nonsteroidal antiinflammatory drugs, and its formation can be modulated at the transcriptional and/or translational levels by antiinflammatory steroids *(8,9)*.

A crucial factor and drawback of PGHs application is the fast and irreversible inactivation of the enzyme in the course of catalysis *(2–4)*. The enzyme inactivation is accompanied by formation of hemoprotein radicals and a relatively slow destruction of the heme–enzyme complex *(10,11)*. It has been demonstrated that fast and dramatic changes in the protein structure occur in the course of the substrate conversion *(12)*. The kinetic experiments and theoretical analysis *(13)* demonstrated that the inactivation proceeds via enzyme–substrate intermediates involved in the mechanism of AA conversion into PGH_2 *(4,5)*.

PGHs is an integral protein located in cells, mainly in the membrane of the endoplasmic reticulum *(2–5)*. The enzyme is commonly obtained by precipitation of microsomal fraction using ultracentrifugation. The microsomal fraction is used for research purposes and as a source of the enzyme *(14,15)*. PGHs preparations from microsomes are fairly stable in solution *(16)*. However, the enzyme is rapidly inactivated in the complex with heme in the presence of oxygen. The inactivation can be avoided by the addition of an electron donor *(4)*.

To apply PGHs for AA determination one needs to obtain the enzymatic preparation with high specific activity and to use an electron donor permitting high sensitivity monitoring.

In this paper we report on:

1. The comparison of PGHs activity and stability of solubilized preparations obtained from bovine vesicular glands by ultracentrifugation or application of Ca^{2+} ions in deoxygenated and nondeoxygenated buffers.
2. Application of different electron donors (adrenaline, ABTS, luminol) in the reaction catalyzed by PGHs from bovine and sheep vesicular glands.

Materials and Methods

Preparations of PGHs were isolated from bovine and sheep vesicular glands according to different methods applying the capacity of calcium ions or ultracentrifugation to precipitate microsomes as described previously *(14,17,18)*. To obtain the PGHs preparations from bovine vesicular glands by both techniques, deoxygenated and nondeoxygenated buffers were used. Deoxygenation was performed using a vacuum pump for 1 h. The microsomal fraction solubilized in Tween-20 was used in the experiments with different electron donors. The Beckman J2-HS and XL-90 centrifuges with rotors JA-10 and 55.2 Ti, respectively, were used. The following reagents were purchased from Sigma Chemical Company (St. Louis, MO): Trizma, diethyldithiocarbamate sodium salt (DEDTC), Tween-20, $CaCl_2$, $NaClO_4$, HCl, arachidonic acid, L-adrenaline, hemine, 2,2'-azino-bis (3-ethylbenz-thiazoline-6-sulfonic) acid (ABTS), 5-amino-2,3-dihydrophthalazine-1,4-dione (luminol), *para*-iodophenol. EDTA disodium salt, hydrogen peroxide (H_2O_2), and ethanol were from Baker (USA).

The protein concentration was determined according to the method of Lowry *(19)*. Spectrophotometric assay *(14)* of PGHs cyclo-oxygenase plus peroxidase activities was performed in 2 mL of Tris-HCl buffer, pH 8.0, containing 0.05% of Tween-20, adding the arachidonic acid (50 µL of 15 m*M* solution in ethanol), L-adrenaline (30 µL of 100 m*M*) as an electron donor, hemine (20 µL of 20 µ*M* and 20 µL of 200 µ*M* for bovine and sheep enzyme preparation, respectively) as a prosthetic group. The optimal concentrations of hemine were determined in additional experiments by spectrophotometric titration with hemine (the results not shown). Kinetics of L-adrenaline oxidation in the PGHs catalyzed reaction was monitored at 480 nm (Beckman DU-50) at 22°C. Kinetics of ABTS oxidation was followed at 405 nm at the same temperature. The activity was estimated as the reaction rate measured in the first seconds after mixing and divided by the protein concentration of PGHs preparation used. Initial rates of the PGHs catalyzed reaction were determined for different concentrations of arachidonic acid in the presence of L-adrenaline and ABTS using PGHs preparations from bovine and sheep vesicular glands.

PGHs solubilized preparation from bovine vesicular glands was applied to detect the enhanced chemiluminescent intensity in the reaction of luminol peroxidation in the presence or in the absence of hydrogen per-

oxide. The reaction conditions were similar to those described above but adrenaline was substituted by luminol and *para*-iodophenol solution (0.01 and 0.03 m*M*, respectively). To obtain the calibration plot for AA, hydrogen peroxide (0.015%) was injected into a luminometer cuvet after 1.5 min of PGHs reaction with a fixed volume of AA solution. The maximum value of chemiluminescent intensity was recorded. The same procedures were performed using the same volume of ethanol instead of AA. The difference in the maximum values of intensity was plotted as a function of AA concentration.

Results and Discussion

It was shown earlier *(4,5)* that the PGHs–hemine complex is rapidly inactivated in the presence of oxygen. To prevent the inactivation the addition of an electron donor is commonly used. The present work demonstrates that elimination of oxygen from the buffer solutions before PGHs extraction also protects enzyme against the inactivation.

The extraction methods used were based on precipitation of microsomal fractions by centrifugation (during 20 min) at 17,000*g* in the Ca (II) ion presence *(17,20)* or ultracentrifugation (during 90 min) at 130,000*g*. The scheme includes the treatment of microsomes with "chaotropic" agent ($NaClO_4$), which was used to remove some proteins other than PGHs from a particulate fraction. Tables 1 and 2 give an example of the results obtained during the PGHs extraction using two mentioned techniques and deoxygenated and nondeoxygenated buffers. The effect of oxygen removal was observed at each step of PGHs extraction (*see also* Table 3). However, the combination of $NaClO_4$ with Ca^{2+} treatment results in a lower specific activity of enzyme, probably owing to the partial enzyme inactivation. Thus, the magnitude of effect of oxygen removal on the enzyme specific activity was higher in the case of ultracentrifugation than in the case of Ca^{2+} treatment. The higher PGHs activity was detected for solubilized microsomal fraction obtained by ultracentrifugation technique using deoxygenated buffers. However, the ultracentrifugation technique requires more time and expensive equipment. The preparation volume treated in the ultracentrifuge is smaller than that in the common centrifuge. Thus, the method with Ca^{2+} treatment is more efficient compared to the ultracentrifugation method.

At the same time, we observed that PGHs of solubilized microsomal fraction from bovine vesicular glands obtained in the presence of Ca^{2+} ions was more stable than the preparations obtained by ultracentrifugation. Table 4 presents the results obtained on the enzyme storage stability at –15°C. The stability of preparations obtained using the deoxygenated buffers was similar to that of the preparations obtained without deoxygenation. The results obtained in this work are in agreement with the results of Mevkh et al. *(16)* who reported that the PGHs of microsomal fraction obtained by ultracentrifugation lost 70% of activity in 72 h. Figure 1 demonstrates

Table 1
Purification Scheme of PGH-Synthase from Bovine Vesicular Glands by Ca^{2+} Treatment Using Deoxygenated (1) and Nondeoxygenated (2) Buffers

	Total protein, mg		Specific activity, mmol/mg/min		Total activity, mmol/min	
Preparation	1	2	1	2	1	2
17,900*g* supernatant	2613	3180	7.07×10^{-3}	3.1×10^{-3}	18.4	9.9
Microsomes precipitated by Ca^{2+}	272	216	11.1×10^{-3}	9.6×10^{-3}	3.0	2.1
$NaClO_4$-washed microsomes	170	200	8.2×10^{-3}	6.1×10^{-3}	1.4	1.2
Tween-20 solubilized microsomes	64	52	2.1×10^{-2}	1.7×10^{-2}	1.3	0.9

Table 2
Purification Scheme of PGH-Synthase from Bovine Vesicular Glands by Differential Centrifugation Using Deoxygenated (1) and Nondeoxygenated (2) Buffers

	Total protein, mg		Specific activity, mmol/mg/min		Total activity, mmol/min	
Preparation	1	2	1	2	1	2
17,900*g* supernatant	750	867	7.07×10^{-3}	3.1×10^{-3}	5.3	2.68
Microsomes precipitated by Ca^{2+}	85	75	2.0×10^{-2}	1.6×10^{-2}	1.7	1.2
$NaCLO_4$-washed microsomes	53	50	2.3×10^{-2}	1.8×10^{-2}	1.2	0.9
Tween-20 solubilized microsomes	39	44	3.1×10^{-2}	1.9×10^{-2}	1.2	0.8

that the PGHs preparation obtained in the presence of Ca^{2+} ions preserves 60% of its activity during two weeks of storage at –15°C.

The solubilized microsomal fractions from bovine and sheep vesicular glands obtained in the presence of Ca^{2+} ions were used to obtain the calibration graph (Fig. 2) for AA determination by means of spectrophotometric technique and adrenaline as an electron donor. Table 5 presents the evaluation data calculated from calibration graphs. The calibration graphs present a linear dependence over the range of 10–70 μ*M* and of 2–50 μ*M* concentrations of AA for bovine and sheep PGHs, respectively. The detection limit was 10 μ*M* and 2 μ*M*, respectively. RSD was <5% ($n = 6$) for these AA concentrations.

Table 3
Relative Specific Activity of PGH-Synthase Preparations Obtained from Bovine Vesicular Glands by Different Methods Using Deoxygenated (1) and Nondeoxygenated (2) Buffers

	By Ca^{2+} treatment (%)		By differential centrifugation (%)	
Preparation	1	2	1	2
17,900*g* supernatant	34	15	23	10
Precipitated microsomes	53	45	65	52
$NaCLO_4$-washed microsomes	39	29	74	58
Tween-20 solubilized microsomes	100	81	100	61

The specific activities of solubilized preparations obtained by each method were used as 100%.

Table 4
Storage Stability (–15°C) of PGHs Preparations Obtained from Bovine Vesicular Glands Using Deoxygenated (1) and Nondeoxygenated (2) Buffers

	Specific activity of preparations							
	Obtained by Ca^{2+} treatment				Obtained by ultracentrifugation			
	1		2		1		2	
Storage, d	*	%	*	%	*	%	*	%
0	1.18	100	1.70	100	1.90	100	3.16	100
8	0.97	82	1.45	85	0.63	33	1.04	33
12	0.73	62	1.09	64	0.55	29	0.95	30

*Specific activity is expressed as μmol/10^2 product formed per minute per milligram protein.

According to the theoretical model described by Varfolomeev and Mevkh *(4,5,20)* and using the kinetic curves of adrenaline oxidation catalyzed by PGHs we figured out the dependence of the rate constant ($k_{in\ app}$) of enzyme inactivation in the course of the reaction as the function of inverse AA concentration (Fig. 3 B). Figure 3 demonstrates that $k_{in\ app}$ for both PGHs from bovine and sheep vesicular glands depends linearly on AA inverse concentration. The tangents of the lines may be interpreted as the ratio of individual parameters of inactivation and were equal for both cases. However, the values of $k_{in\ app}$ and individual k_{in} (determined from intercepts on ordinate axis) for PGHs from bovine vesicular glands are higher than the same values determined for the enzyme from sheep vesicular glands (Fig. 3A).

To calculate $k_{in\ app}$ the time dependence of substrate conversion degree [$\alpha = P/S_0 = \alpha_{lim}(1 - e^{-k\ in\ app\ t})$ where P is product, S_0 is initial concentration of substrate] was analyzed. The values of $k_{in\ app}$ were calculated as the tangent of the lines in the semi-logarithmic plot ($\ln[1 - \alpha/\alpha_{lim}]$ vs time) obtained for different AA concentrations. It was demonstrated that the dependence

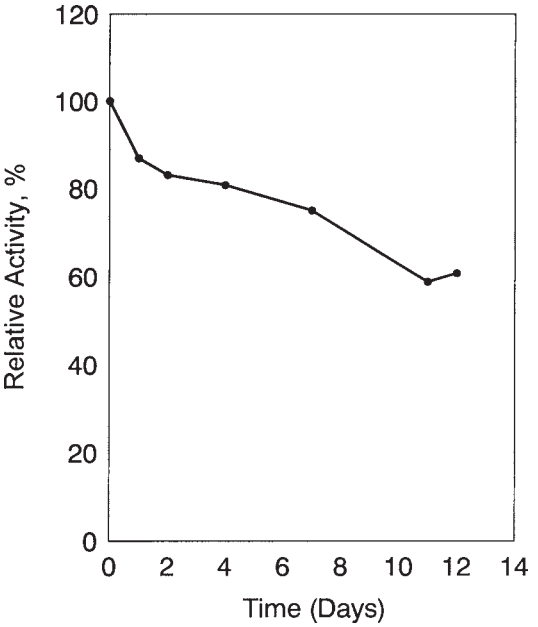

Fig. 1. Storage stability (–15°C) of PGH-synthase from a solubilized microsomal preparation obtained from bovine vesicular glands by Ca^{2+} treatment.

of maximal yield of the product (α_{lim}) on the initial AA concentration also may be used as a calibration plot for AA determination (Fig. 4 and Table 5).

Then, ABTS was applied as an electron donor in the PGHs catalyzed transformation of AA. The oxidation of ABTS was not observed in the case of PGHs from bovine vesicular glands. However, it can be used to obtain the calibration plot for AA determination using the reaction catalyzed by PGHs from sheep vesicular glands (Fig. 5 and Table 5). This result may be explained by the differences in catalytic activity and in the structure of these enzymes as well as their different affinity for the electron donor of distinct nature.

Using luminol in the reaction of AA transformation catalyzed by PGHs from bovine vesicular glands we could not detect the chemiluminescent intensity of luminol peroxidation reaction. However, light emission was detected when hydrogen peroxide was added. The value of maximum chemiluminescent intensity was sensitive to the presence of ethanol (Fig. 6, curve 1) that was used as a solvent for AA standard solutions.

According to the data of different authors *(10,11,21)*, the inactivation of cyclo-oxygenase activity in the reaction of AA transformation catalyzed by PGHs is 2–3 times faster than peroxidase activity. On the basis of this observation, we decided to detect the chemiluminescent intensity of luminol peroxidation reaction after the fixed time of AA transformation. We observed that the maximum intensity of chemiluminescence of luminol peroxidation decreased with increasing of AA concentration in the system (Fig. 6, curve 2). The calibration plot in Fig. 7 was obtained from the data in Fig. 6 and shows the percentage of maximum intensity of chemilumines-

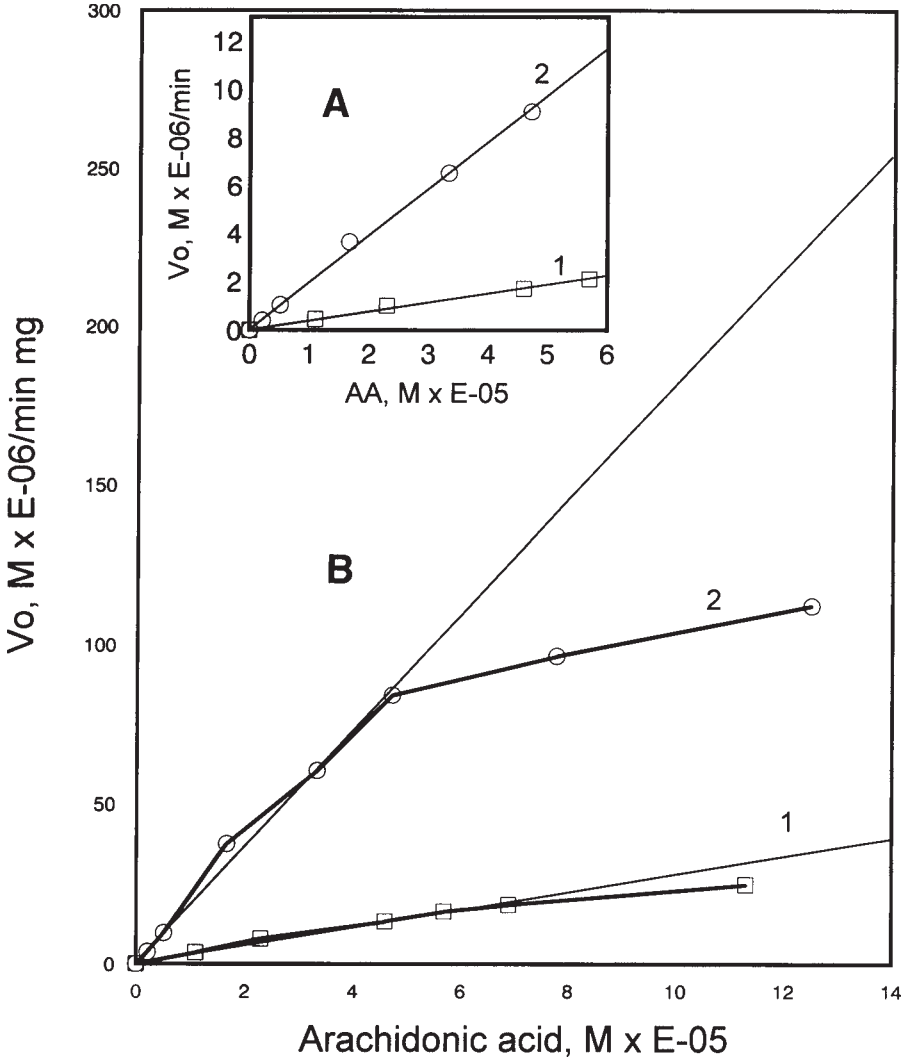

Fig. 2. Calibration graphs for AA determination using L-adrenaline in the presence of PGHs from bovine vesicular glands (1) and PGHs from sheep vesicular glands (2). Reaction condition: L-adrenaline 1.37 m*M*; hemine 0.2 μ*M* and 4.5 μ*M*, respectively for bovine and sheep enzyme preparation; buffer Tris-HCl 50 m*M*, pH 8.0, Tween-20 0.05%; temperature 22°C. Initial rate of the AA transformation process **(A)** and initial rate per mg of protein as the functions of AA concentration **(B)**.

cence of luminol peroxidation in the system with ethanol and AA (*see also* Table 5). There are two possible reasons for the observed phenomenon of inhibition. One may account for partial inactivation of PGHs in the course of AA oxidation in the absence of H_2O_2. The second variant could be a common inhibition of peroxidase-catalyzed luminol peroxidation in the presence of AA. To discriminate between these two possibilities an additional study has to be performed.

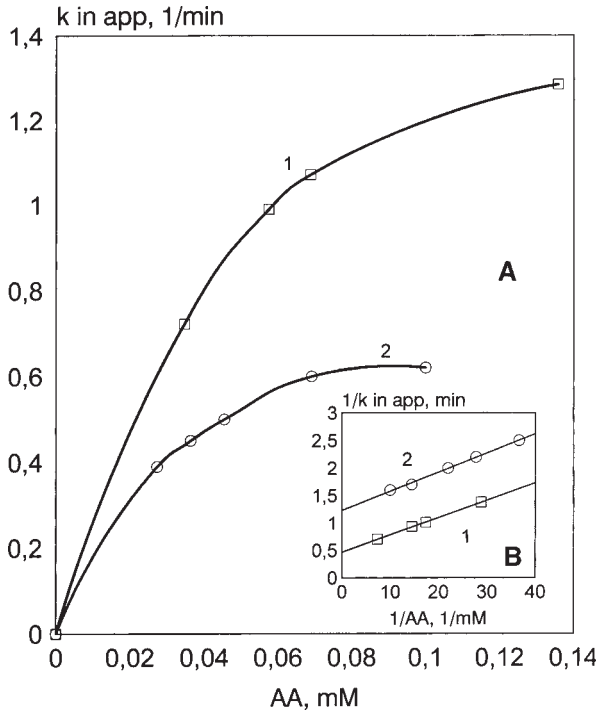

Fig. 3. Inactivation constants of PGHs from bovine vesicular glands (1) and from sheep vesicular glands (2) as a function of AA concentration in direct **(A)** and inverse **(B)** coordinates.

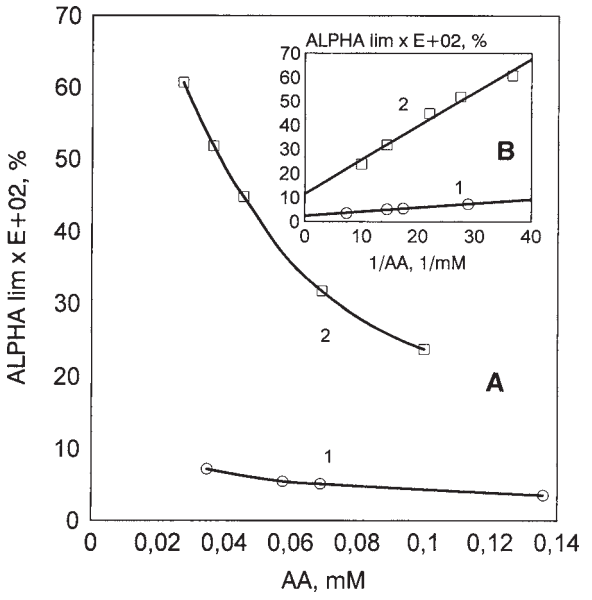

Fig. 4. Calibration graphs for AA determination using L-adrenaline (1.37 m*M*) in the presence of PGHs from bovine vesicular glands (1) and PGHs from sheep vesicular glands (2), with α_{lim} as calculated parameter in direct **(A)** and semi-inverse **(B)** coordinates.

Table 5
Evaluation Data of Arachidonic Acid Determination
by Means of Different Calibration Plots

Electron donor/plot parameters/ source of enzyme	Minimal value, μ*M*	Linear range, μ*M*	Correlation coefficient	*R*-Squared
Adrenaline/(V_0 vs AA)/bovine glands	10	1–70	0.997	0.995
Adrenaline/(V_0 vs AA)/sheep glands	2	2–50	0.997	0.994
Adrenaline/(α_{lim} vs 1/AA)/bovine glands	10	10–135	0.990	0.981
Adrenaline/(α_{lim} vs 1/AA)/sheep glands	2	2–120	0.993	0.988
ABTS/(V_0 vs AA)/sheep glands	0.3	0.3–30	0.999	0.999
Luminol/(% I_{max} vs AA)/bovine glands	1	1–400	–0.998	0.997

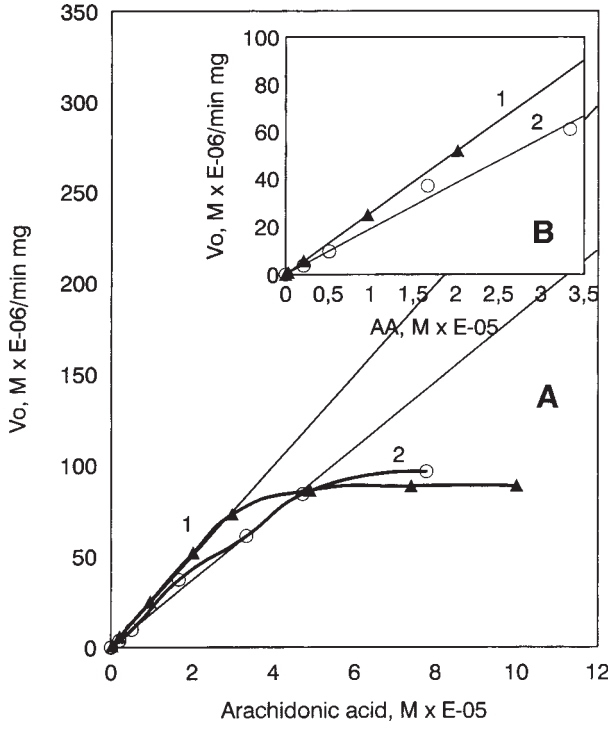

Fig. 5. Calibration graphs for AA determination obtained using ABTS (1) and L-adrenaline (2) in the presence of PGHs from sheep vesicular glands: a whole curves **(A)**; an initial part of the same curves **(B)**. The concentration of the both electron donors is 1.37 m*M*.

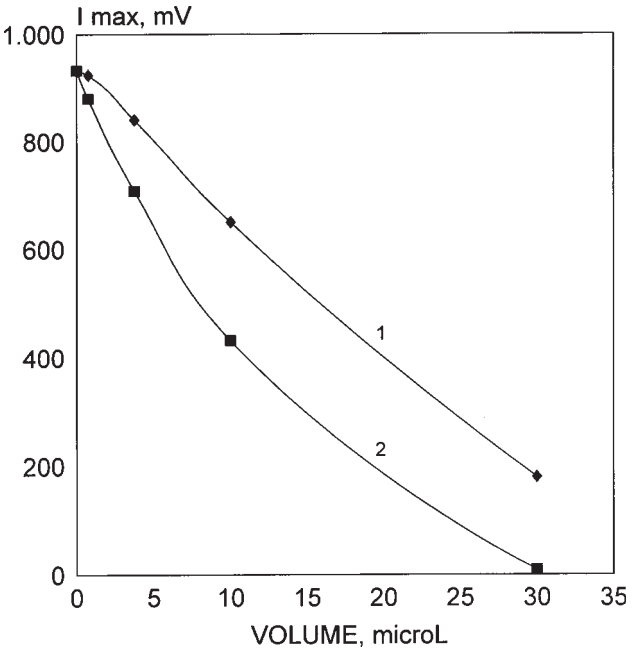

Fig. 6. Maximum intensity of chemiluminescence of luminol peroxidation detected in the system of AA transformation catalyzed by PGHs from bovine vesicular glands: as a function of a volume of ethanol added in the system as a control (1); as a function of a volume of AA solution in ethanol added to the system (2).

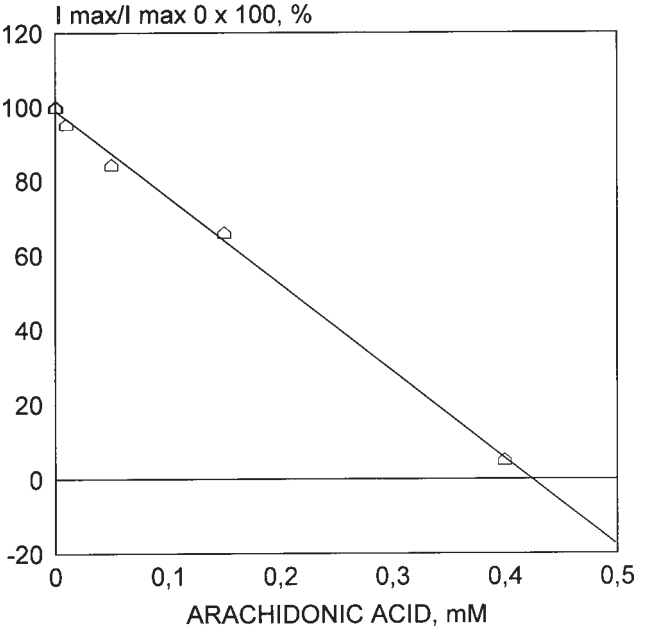

Fig. 7. Calibration graph for AA determination obtained from data presented in Fig. 6 for the system of enhanced chemiluminescent reaction of luminol peroxidation.

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