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Enzyme Self-Inactivation Is a Main Limitation of the Preparation of Eicosanoids

Enzymatic Synthesis of Thromboxane and 12(S)-Hydroxytetraenoic Acid

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Abstract

Synthesis of prostanoids is accompanied by various processes reducing the product yield. These processes are also known to affect syntheses of thromboxane (TX) and 12(*S*)-hydroxy-5(*Z*),8(*Z*),10(*E*),14(*Z*)-eicosatetraenoic acid (12-HETE). Partially purified preparations of TX synthase and prostaglandin (PG) synthase were used to optimize TX synthesis with respect to concentrations of the enzymes and eicosapolyenoic acid (EPA). Conditions for the maximum product yield and the minimum consumption of enzymes were determined. Consumption of the TX synthase was large owing to its inactivation during the reaction and the nonenzymatic destruction of the intermediate product PG-endoperoxide. Separate addition of PG and TX synthases increased the product yield by preventing EPA sorption on ballast proteins. Microsomal 12-lipoxygenase (12-LO) was also shown to be inactivated during the reaction, and this process was the major limitation of 12-HETE synthesis. Lipoxygenase reaction in the presence of some reducing agents led to a considerable increase of the 12-HETE yield, supposedly by preventing further oxidation of the 12-LO reaction product 12-hydroperoxy derivative of eicosatetraenoic acid. The possibility of using human blood platelet microsomes for preparation of some derivatives of EPAs is discussed.

Index Entries: Eicosanoids; thromboxane; 12-HETE; prostaglandin synthase; thromboxane synthase; 12-lipoxygenase; biosynthesis; enzyme self-inactivation.

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Introduction

Thromboxane B_2 (TBX_2) and 12(*S*)-hydroxy-5(*Z*),8(*Z*),10(*E*),14(*Z*)-eicosatetraenoic acid (12-HETE) were first discovered by analysis of products of incubation of radioactively labeled arachidonic acid with human blood platelets *(1)*. These compounds are the stable forms of enzymatically formed thromboxane A_2 (TXA_2) and 12(*S*)-hydroperoxy-5(*Z*),8(*Z*),10(*E*),14(*Z*)-eicosatetraenoic acid (12-HpETE). TXA is formed via isomerization of prostaglandin (PG) endoperoxide *(1,2)*, which is catalyzed by TX synthase (EC 5.3.99.5), whereas 12-HpETE is formed directly from arachidonic acid under the action of arachidonic acid: 12-lipoxygenase (12-LO) (EC 1.13.11.31) (Scheme 1).

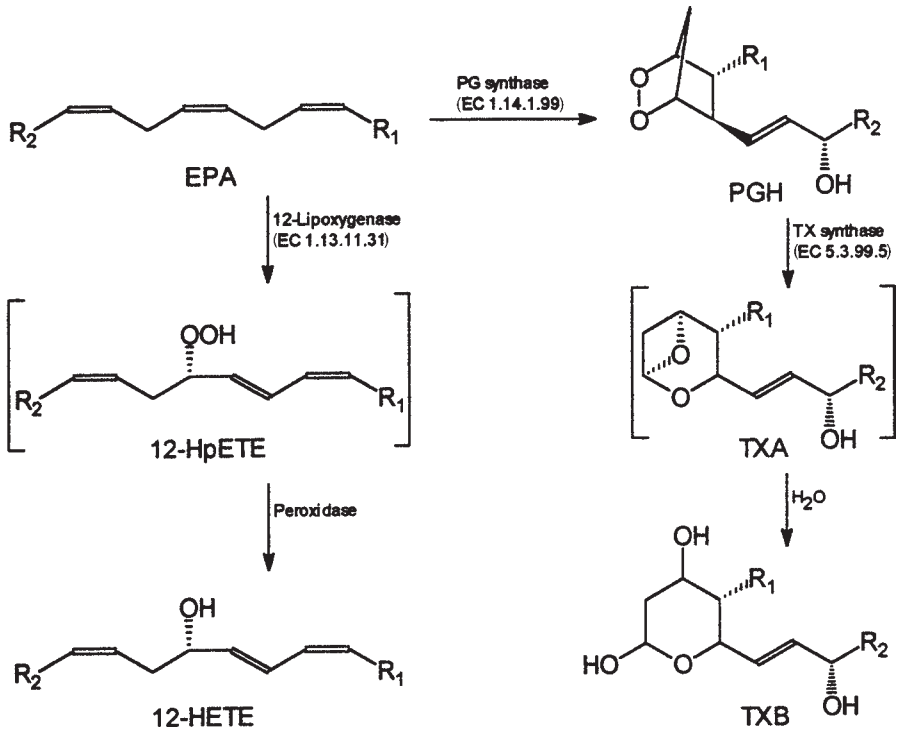
The physiological role of these compounds is well studied. TXA_2 induces the aggregation of blood platelets *(2,3)* as well as the constriction of the smooth muscles of blood vessels and the respiratory tract *(2–4)*. It is also involved in some pathophysiological processes (for a review *see* refs. *5* and *6*). 12-HETE causes chemotaxis of blood cells (reviewed in ref. *7*). It is also known to take part in the metastatic spreading of some cancer cells *(8)*. In addition, 12-HpETE is a precursor in the synthesis of hepoxylines, which have a wide range of biological activities *(9)*.

TXA is an extremely unstable compound. In water medium, it converts ($t_{1/2}$ about 30 s at 37°C) to the biologically inactive product TXB *(2)*. A distinct feature of the TX synthase reaction is that, in its course, several compounds are formed. The composition of the products depends on which PG-endoperoxide is used as a substrate *(1,10,11)*. Prostaglandin H_2 (PGH_2) is converted by TX synthase to TXB_2 and 12(*S*)-hydroxy-(5*Z*,8*Z*,11*E*)-heptadecatrienoic acid (HHT); their ratio is about 1:1 *(10–12)*. In addition to platelets, high TX synthase activity was also discovered in the gastroenteric tract, lungs, kidneys, and other tissues *(13)*.

TX synthase is a membrane microsomal enzyme of the cytochrome P450 type *(11,12,14)*. The homogeneous enzyme was isolated from human blood platelets *(12)* and pig lung *(15)*. TX synthase of various degrees of purity as well as in intact blood platelets is irreversibly inactivated in the reaction *(15–18)* the same way as PG synthase (reviewed in ref. *19*). In addition, TX synthase is irreversibly inhibited by peroxides of fatty acids such as 15-HpETE *(18)*. Supposedly, TXA synthesis is a limiting step of the primary aggregation of blood platelets *(20)*.

Homogeneous 12-LO was isolated from human blood platelets *(21)* and pig leukocytes *(22)*. Those enzymes belong to two different types of 12-LO (for a review *see* ref. *23*). 12-LO is a multifunctional enzyme catalyzing lipoxygenase as well as other reactions *(23)*, in particular 15-HpETE dehydration to 14,15-leukotriene A_4 *(24)*. In human blood platelets, 12-LO is localized in both cytosolic *(25)* and microsomal *(26)* fractions.

In addition to TX synthase and 12-LO blood platelets, microsomes contain PG synthase that is catalytically identical to the enzyme from sheep vesicular glands (SVGs) *(27–29)*.



Scheme 1. Biosynthetic pathways of 12-HpETE and thromboxane, where R_1: $-CH_2-CH=CH-(CH_2)_3-COOH$

R_2:

For arachidonic acid, PGH_2, TXB_2, 12-HpETE, and 12-HETE - $-(CH_2)_4-CH_3$

For thymnodonic acid, PGH_3, and TXB_3 - $-CH_2-CH=CH-CH_2-COOH$

In a previous article *(30)*, we described PGE enzymatic synthesis from eicosapolyenoic acids (EPAs) with the use of microsomes from SVGs. The present article deals with the enzymatic syntheses of TXB and 12-HETE from EPAs using human blood platelets. We carried out optimization of the syntheses of these compounds taking into account inactivation of the enzymes during the reaction as well as other processes capable of limiting the synthesis of the end products.

Materials and Methods

Materials

Arachidonic acid was purchased from Fluka Chemie AG (Buchs, Switzerland). Thymnodonic acid was kindly supplied by Dr. N. A. Latyshev (Institute for Marine Biology, Vladivostok, Russia). The PGs were obtained from the Institute of Chemistry of Estonian Academy of Sciences (Tallinn, Estonia); TXB_2, indomethacin, hemin, and bovine serum albumin (fraction V) from Sigma (St. Louis, MO); L-glutathione from Reanal; L-adrenaline from Serva Feinbiochemica GmbH (Heidelberg, Germany); and DEAE-

cellulose from Whatman Ltd. (Maidstone, England). Tritium-labeled arachidonic and thymnodonic acids were produced by Drs. I. Yu. Nagayev and V. P. Shevchenko (Institute for Molecular Genetics, Moscow, Russia) as described in ref. *31*. Tritium-labeled and nonlabeled prostaglandins H_2 were produced as described in ref. *32*. Other reagents and solvents were analytical grade product. SVGs were kindly provided by Dr. G. I. Myagkova (Moscow State Academy for Fine Chemical Technology, Moscow, Russia).

Preparation of Enzymes

Partially purified PG synthase was produced from SVG microsomes as described previously *(30)*. Human blood platelet microsomes were isolated from the platelet mass (centrifuged blood platelets) by the procedure described in ref. *12*. The platelet mass was received at the Moscow Central Station for Hemotransfusion. It was a byproduct of the production of human plasma proteins. TX synthase preparation (DEAE cellulose fraction) was produced from human blood platelet microsomes using procedures described in refs. *12* and *16*. As a result, 60 g of the platelet mass yielded 2.96 U of TX synthase activity.

Enzyme Assays

PG synthase activity was assayed polarographically as described in ref. *30*. TX synthase activity was assayed by conversion of tritium-labeled PGH_2 to TX using the same procedure as for the PGE synthase assay (*see* ref. *30*). The only difference was that the reaction was performed in 50 m*M* potassium phosphate buffer, pH 7.4. One unit of TX synthase activity was defined as the quantity of the enzymatic preparation converting 1 µmol of PGH_2/min at 32°C.

12-LO activity was assayed by conversion of tritium-labeled arachidonic acid. The standard reaction mixture (200 µL) contained a 12-LO preparation and tritium-labeled arachidonic acid (25 µCi, 10 nmol) in 50 m*M* Tris buffer, pH 7.7. The reaction was carried out for 1 min with constant stirring at 32°C. The reaction was terminated by adding 2 *N* acetic acid to pH 3.0. The reaction products were extracted by ethyl acetate (three times, 0.5 mL each) and analyzed by thin-layer chromatography (TLC) in the solvent system benzene/ethyl acetate/acetic acid (80:20:0.2, v/v/v) on silica gel plates. Distribution of radioactive products was determined with the help of a Berthold LB 2832 radioactivity scanner (Berthold Instrument Inc., Wildbad, Germany). Nonradioactive standards were detected by treating the plates with phosphomolybdenic acid in ethanol. The quantity of the enzymatic preparation converting 1 µmol of arachidonic acid/min at 32°C was taken as 1 U of 12-LO activity.

TX Synthesis from EPAs

To run the enzymatic synthesis of a TX reaction mixture (1 mL) containing enzymatic preparations (microsomes or partially purified enzymatic preparation), phenol (2 m*M*) and hemin (2 µ*M*) in 50 m*M* potassium

phosphate buffer, pH 7.4, was placed into a 15-mL glass vial. After 5 min of preincubation at 25°C, labeled (50 μCi per probe) and nonlabeled EPAs were added. On 1 h of incubation at 25°C, 150 μL of 2 *M* citric acid were introduced into the reaction mixture. The reaction products were extracted by ethyl acetate (two times, 5 mL each). The extracts were combined and then dried with Na_2SO_4, and the organic solvent was evaporated. The residue was dissolved in 20 μL of methanol and a small aliquot (4–5 μL) was loaded on a silica gel plate. TLC was conducted in the solvent system chloroform/methanol/acetic acid (90:9:1, v/v/v). Radioactive products and standards were detected as described above.

Enzymatic synthesis of 12-HETE was conducted likewise except that the reaction mixture contained no phenol and hemin. To examine the effects of various agents on 12-HETE synthesis, the agents were preincubated with the enzymatic preparation before the addition of arachidonic acid. The solvent system benzene/ethyl acetate/acetic acid (80:20:0.2, v/v/v) was used for TLC analysis of radioactive products. To measure the 12-HETE quantity in the extract, ultraviolet spectroscopy was used in addition to the TLC. At molecular extinction coefficient of 27,000 $M^{-1} \cdot cm^{-1}$ at 234 nm was used.

From the content of products in the extract, the extreme concentrations of EPAs and TX, $[EPA]_\infty$ and $[TXB]_\infty$, were determined. TX yield Y, conversion degree of EPA α_1, and degree of PGH conversion to TXB α_2 were calculated as follows:

$$Y = ([TXB]_\infty / [EPA]_0) \times 100\% \quad (1)$$

$$\alpha_1 = ([EPA]_0 - [EPA]_\infty)/[EPA]_0 \quad (2)$$

$$\alpha_2 = [TXB]_\infty / ([EPA]_0 - [EPA]_\infty) \quad (3)$$

Protein concentration in enzymatic preparations was determined according to ref. *33* using bovine serum albumin as a standard.

The mathematical model of the two-enzyme TX synthesis is based on the following data.

1. Kinetics of reactions catalyzed by PG and TX synthases are described by the Michaelis–Menten equation *(34,35)*.
2. PG and TX synthases are subject to an irreversible inactivation during the reaction *(17,34)*.
3. TX synthase reaction is inhibited by fatty acids including arachidonic *(36)*. The inhibition is fast, competitive, and reversible.
4. The intermediate product PGH is subject to a spontaneous destruction in water medium *(1)*.

Taking all this into account, the mathematical model of the two-enzyme TX synthesis can be represented by the following system of differential equations:

$$-\frac{d[EPA]}{dt} = \frac{k^I \cdot \{[PgS]_0 - l \cdot (1/v^I) \cdot ([EPA]_0 - [EPA])\} \cdot [EPA]}{K_M^I + [EPA]}$$

$$\frac{d[\mathrm{PGH}]}{dt} = \frac{k^{\mathrm{I}} \cdot \{[\mathrm{PgS}]_0 - l \cdot (1/\nu^{\mathrm{I}}) \cdot ([\mathrm{EPA}]_0 - [\mathrm{EPA}])\} \cdot [\mathrm{EPA}]}{K_M^{\mathrm{I}} + [\mathrm{EPA}]}$$

$$- \frac{k^{\mathrm{II}} \cdot \{[\mathrm{TxS}]_0 - m \cdot (1/\nu^{\mathrm{II}}) \cdot ([\mathrm{TX}]/\beta)\} \cdot [\mathrm{PGH}]}{K_M^{\mathrm{II}} \cdot \{1 + n \cdot ([\mathrm{EPA}]/K_{\mathrm{I}})\} + [\mathrm{PGH}]} - p \cdot k_{\mathrm{ne}} \cdot [\mathrm{PGH}] \qquad (4)$$

$$\frac{d[\mathrm{TX}]}{dt} = \frac{k^{\mathrm{II}} \cdot \{[\mathrm{TxS}]_0 - m \cdot (1/\nu^{\mathrm{II}}) \cdot ([\mathrm{TX}]/\beta)\} \cdot [\mathrm{PGH}]}{K_M^{\mathrm{II}} \cdot \{1 + n \cdot ([\mathrm{EPA}]/K_{\mathrm{I}})\} + [\mathrm{PGH}]} - p \cdot k_{\mathrm{ne}} \cdot [\mathrm{PGH}]$$

$$\frac{d[\mathrm{P_{ne}}]}{dt} = p \cdot k_{\mathrm{ne}} \cdot [\mathrm{PGH}]$$

in which [EPA], [PGH], [TX], and [P_{ne}] are the concentrations of EPA, PGH, TX synthase products (TX + HHT), and all the products of the nonenzymatic PGH destruction at the time t, respectively; $[PgS]_0$ and $[TxS]_0$ are the initial concentrations of PG and TX synthases, respectively; K_M^{I} and K_M^{II} are the Michaelis constants of PG and TX synthase reactions, respectively; K_I is the equilibrium constant of dissociation of the enzyme-inhibitor complex that is the complex of TX synthase and EPA for our system; k^{I} and k^{II} are the catalytic constants of PG and TX synthases reactions, respectively; k_{ne} is the rate constant for PGH nonenzymatic destruction; ν^{I} and ν^{II} are the coefficients corresponding to the number of PG synthase and TX synthase turnovers before their complete inactivation, ν^{I} and $\nu^{II} >> 1$; β is the fraction of TX in the total sum of enzymatic products formed in the TX synthase reaction; and l, m, n, and p are the coefficients introduced to permit the examination of different variants of the system—they can take values of 0 or 1.

The following values of kinetic parameters of the reactions were used for calculations: K_M^{I}, 5 μM; K_M^{II}, 22 μM; K_I, 22 μM; k^{I}, 5400 min^{-1}; k^{II}, 3200 min^{-1}; k_{ne}, 0.2 min^{-1}; ν^{I}, 4050 for arachidonic acid and 350 for thymnodonic acid; ν^{II}, 32,000; β, 0.5.

Calculations of the variables of the system of differential Eqs. 4 were conducted as described previously *(30)*.

Results

Production of Enzymatic Preparations

PG synthase was obtained from SVGs. Both the microsomal fraction and the partially purified enzyme were used. Protocols of their isolation, activity, stability, and other parameters are described in detail in ref. *30*.

Microsomes of SVGs contain high PGE synthase activity that has a pronounced glutathione dependence *(37)*. Since the incubation was conducted in the absence of reduced glutathione, PGE synthase activity was negligible.

Microsomes extracted from the platelet mass were used as enzymatic preparations of TX and 12-LO synthases. In addition to these two enzymes,

human blood platelet microsomes also contained PG synthase. Having taken into account the activities of these enzymes in the microsomes, we found that 100 g of the platelet mass contained about 130 U of PG synthase activity, about 4 U of TX synthase activity, and about 6 U of 12-LO activity. Storage of the human blood platelet microsomes led to a substantial decrease in the 12-LO activity, whereas, during the same time, the activities of PG and TX synthases did not alter. This was clearly demonstrated by a shift in the ratio of the products of arachidonic acid incubation with human blood platelet microsomes toward the increase in TX fraction. Thus, all studies on optimization and synthesis of 12-HETE were conducted with freshly prepared microsomes from human blood platelets.

A preparation of partially purified TX synthase was also used. It was produced from the microsomes by solubilization of membrane proteins with the nonionic detergent Lubrol PX and their further fractionation on a DEAE cellulose column. The resulting TX synthase preparation did not contain PG synthase activity. The TX synthase activity did not decrease for 3 mo of storage at –60°C.

Conversion of Arachidonic Acid by Human Blood Platelet Microsomes

Incubation of tritium-labeled arachidonic acid with human blood platelet microsomes led to the formation of a number of products, mainly 12-HETE, TXB, and HHT (Fig. 1A,B). The maximum TX yield (about 15%) was achieved at a microsome concentration of 1 to 2 mg/mL, whereas the maximum 12-HETE yield (about 35%) was reached at a microsome concentration of 5 to 6 mg/mL (Fig. 2). The ratio of the products depended on the concentration of the microsomes. An increase in the concentration decreased the fraction of cyclooxygenase products and TXB_2. When the reaction was carried out in the presence of microsomes from SVGs and when PG synthase activity considerably exceeded 12-LO activity, the observed dependencies did not change (data not shown). This indicates that in the conditions of this experiment, TX synthesis by human blood platelet microsomes is not limited by PG synthase activity.

Incubation of human blood platelet microsomes with arachidonic acid in the presence of indomethacin (0.2 m*M*) led to the accumulation of 12-HpETE in the reaction mixture. This is accounted for by an inhibiting effect of indomethacin on peroxidase of human blood platelets *(38)*.

Determination of Stoichiometry of TX Synthase Reaction Products

In the reaction catalyzed by TX synthase, several products are formed from PGH_2 and some other PG-endoperoxides: TXB, HHT, and malonic dialdehyde *(1)*.

Using the available data on the products of two-enzyme TX synthesis from arachidonic acid, the HHT/TXB_2 ratio was determined. An increase

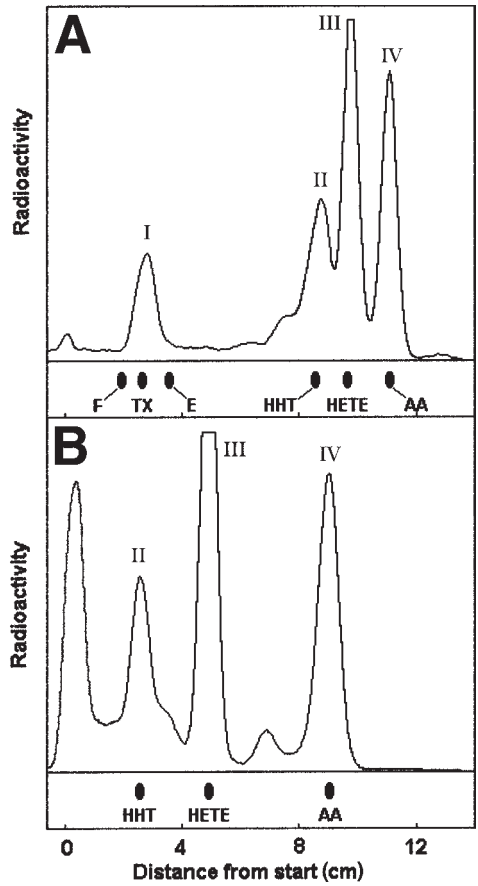


Fig. 1. TLC analysis of radioactive products of tritium-labeled arachidonic acid conversion by human blood platelet microsomes. **(A)** Solvent system chloroform/methanol/acetic acid (90:9:1, v/v/v); **(B)** solvent system benzene/ethylacetate/acetic acid (80:20:0.2, v/v/v). One hundred microliters of the reaction mixture containing human platelet microsomes (0.18 mg), arachidonic acid (5 nmol), tritium-labeled arachidonic acid (25 µCi), phenol (0.2 µmol), and hemin (0.2 nmol) in 50 m*M* potassium phosphate buffer, pH 7.4, was incubated for 1 h at 32°C. F and E, prostaglandins F_2 and E_2, respectively; TX, TXB_2; AA, arachidonic acid. I, II, III and IV are radioactive products identified as TXB_2, HHT, 12-HETE, and arachidonic acid, respectively.

in TX yield led to a decrease in HHT/TXB_2 ratio (data not shown). At a high TX yield (about 20%) this ratio was about 1.4:1.

Effect of Self-Inactivation of Enzymes and Other Processes on TX and 12-HETE Syntheses

Some enzymes of the arachidonic acid cascade including PG and TX synthases are well known to be subject to an irreversible inactivation during the reaction *(15,16,34,39,40)*. Kinetics of the process are described by the Eq. 5:

$$de/ds = 1/\nu \qquad (5)$$

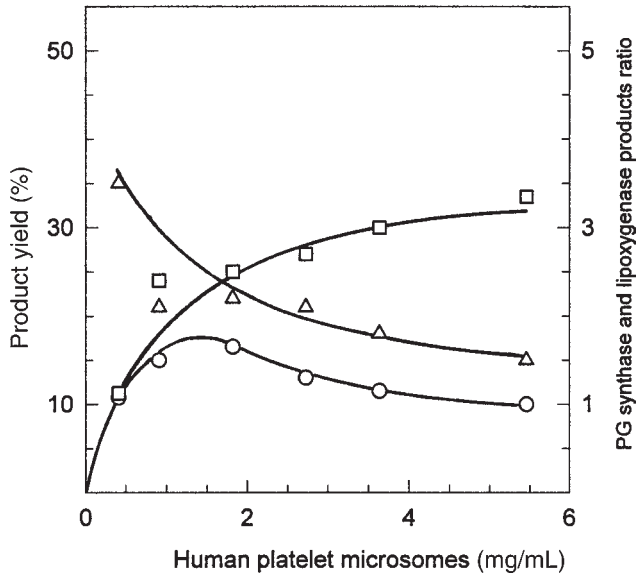


Fig. 2. Conversion of arachidonic acid by human blood platelet microsomes. Reaction conditions are the same as in Fig. 1. (—○—), TXB_2 yield; (—□—), 12-HETE yield; (—△—), ratio of concentrations of PG synthase and lipoxygenase products.

Table 1
Number of Enzyme Turnovers Before Complete Inactivation (ν) for Enzymes of TX and 12-HETE Syntheses

		Maximum product yield	
Enzyme	Substrate	nmol/mU[a]	ν
PG synthase	Arachidonic acid	0.75	4040[b]
	Thymnodonic acid	0.06	350[b]
TX synthase	PGH_2	9.00	6700
12-LO	Arachidonic acid	6.50	—

[a]U, units of enzyme activity.
[b]Results reproduced with permission from ref. *30*.

in which *e* and *s* are the current concentrations of enzyme and substrate, respectively; and ν is the number of the enzyme turnovers before complete inactivation.

The ν value can be estimated by determining the change in the enzyme activity depending on the quantity of the reaction product formed. Table 1 presents experimental results allowing estimation of the ν value.

Influence of the PG synthase inactivation on the two-enzyme synthesis of prostanoids is considered in detail in ref. *30*. This influence can be observed only in excess of EPA, i.e., at $[EPA]_0 > \nu^I \cdot [PgS]_0$. In this case the product yield is reduced owing to both an incomplete conversion of EPA and inhibition of PGH-converting enzyme by the free EPA.

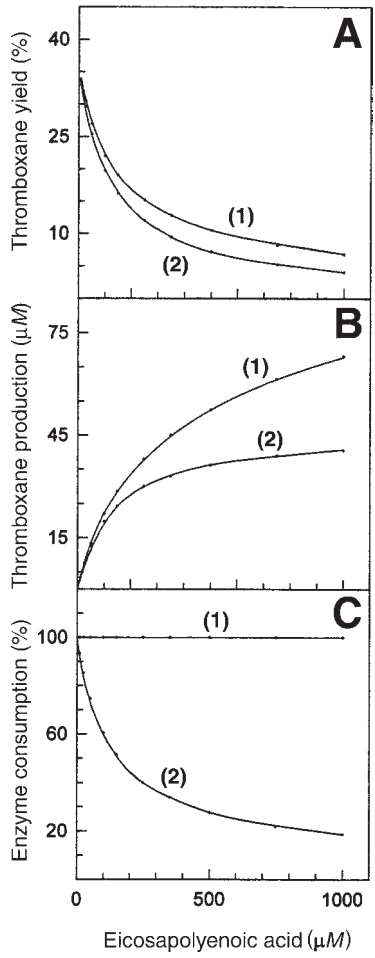


Fig. 3. Effect of EPA concentration on a TX yield **(A)**, extreme TX concentration **(B)**, and degree of the enzyme consumption **(C)** calculated for the two-enzyme model of TX synthesis (*see* Eq. 4) for systems with a stable (1) and an inactivated (2) TX synthase.

TX synthase reaction runs on the nonenzymatic efflux of the substrate. The effect of TX synthase inactivation on TX synthesis is especially strong at high EPA concentrations (Fig. 3A). The maximum concentration of the product reaches its extreme value (equal to $v^{II}\cdot[TxS]_0$) at $[EPA]_0 >> v^{II}\cdot[TxS]_0$ and $[EPA]_0 \geq v^{I}\cdot[PgS]_0$ (Fig. 3B). In these conditions, most of the TX synthase is inactivated (Fig. 3C). The dependence of the degree of TX synthase inactivation on EPA concentration is nonlinear. This is connected to the nonenzymatic destruction of an intermediate product.

Analysis of experimental results demonstrated that even at high TX synthase concentrations, when the inactivation should not considerably influence TX synthesis, the product yield did not reach the maximum values. This showed that the inactivation of the enzymes was accompanied by other processes reducing the yield of the products. These processes were a

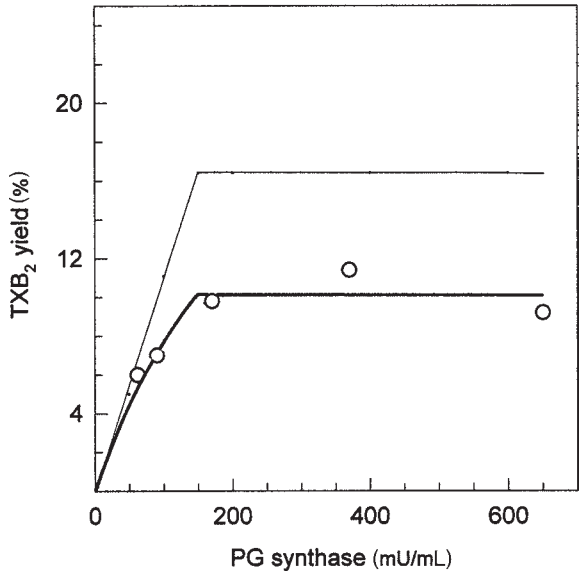


Fig. 4. Effect of PG synthase concentration on TXB_2 synthesis. Arachidonic acid, 100 μ*M*; TX synthase, 8 mU/mL. (—○—), Experimental data; (——), calculated data.

nonenzymatic destruction of the intermediate product, sorption of EPAs on proteins, and inhibition of the TX synthase reaction by EPAs. The pattern of the influence of these processes on TX synthesis was similar to that observed in the enzymatic PGE synthesis (*see* ref. *30*).

Optimization of TX Synthesis

Determination of Conditions for Maximum Product Yield

Conditions for maximum product yield in the two-enzyme TX synthesis were determined using partially purified PG synthase and TX synthase preparations (Figs. 4–6).

With an increase in PG synthase concentration, TX yield grew to a certain value after which it did not alter (Fig. 4). Calculations based on the mathematical model showed that the dependence of TX yield on PG synthase concentration had two separate regions. At $[PgS]_0 < [EPA]_0/\nu^I$, TX yield depended linearly on the PG synthase concentration, whereas at $[PgS]_0 \geq [EPA]_0/\nu^I$, TX yield reached a maximum value and became independent of the PG synthase concentration.

TX yield depended on the TX synthase concentration and was higher when arachidonic acid was used as an initial compound (Fig. 5A,B). At high TX synthase concentrations TX yield did not reach the extreme values. This was owing to a nonspecific PGH conversion by ballast proteins present in TX synthase preparation, as was observed in the PGE synthesis *(30)*. Results obtained with the mathematical model showed that TX yield increased with TX synthase concentration and that this dependence was nonlinear

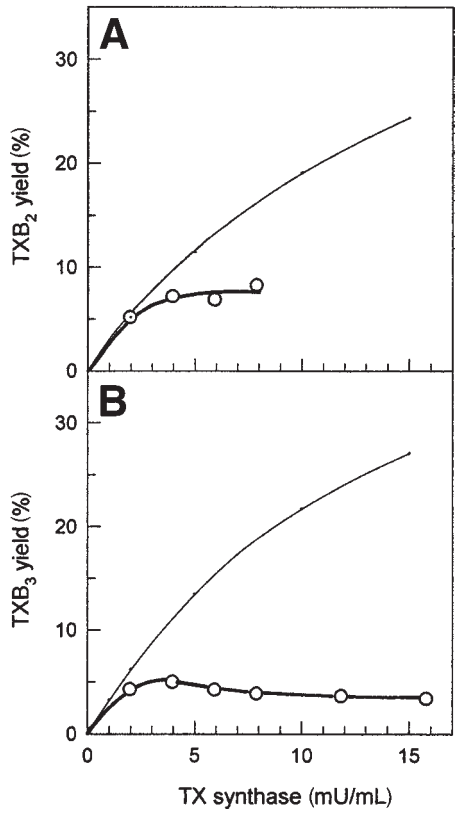


Fig. 5. Effect of TX synthase concentration on synthesis of TXB_2 **(A)** and TXB_3 **(B)**. (A) Arachidonic acid, 100 μ*M*; PG synthase, 0.12 U/mL. (B) Thymnodonic acid, 75 μ*M*; PG synthase, 0.4 U/mL. (—○—), Experimental data; (——), calculated data.

(Fig. 5A,B), because PGH converts by two pathways: enzymatic and nonenzymatic. At $[TxS]_0 >> (k_{ne}/k^{II}) \cdot K_M^{II}$, TX yield reaches a value of $\beta \cdot \alpha_1 \cdot 100\%$. TX synthase inactivation affects TX yield only at low enzyme concentrations when $[TxS]_0 << [EPA]_0/\nu^{II}$ (*see also* Fig. 3C).

TX yield also depended on EPA concentration (Fig. 6A). The maximum yield was reached at low (about 50 μ*M*) concentrations of arachidonic acid. An increase in its concentration reduced TX yield and also decreased efficiencies of the PG synthase and TX synthase reactions (Fig. 6B,C). Similar results were obtained using the mathematical model. The dependence of TX yield on the EPA concentration had a fracture at $[EPA]_0 = \nu^{I} \cdot [PgS]_0$. The maximum product yield was reached at $[EPA]_0 << 3 \cdot K_M^{II}$ and $[EPA]_0 \leq \nu^{I} \cdot [PgS]_0$. A decrease in TX yield with an increase in EPA concentration was owing to a nonenzymatic destruction of the intermediate product and TX synthase inactivation, and when $[EPA]_0 > \nu^{I} \cdot [PgS]_0$, it was also owing to the PG synthase inactivation and TX synthase inhibition by EPA.

The studies just described enabled us to determine conditions for the maximum TX yield:

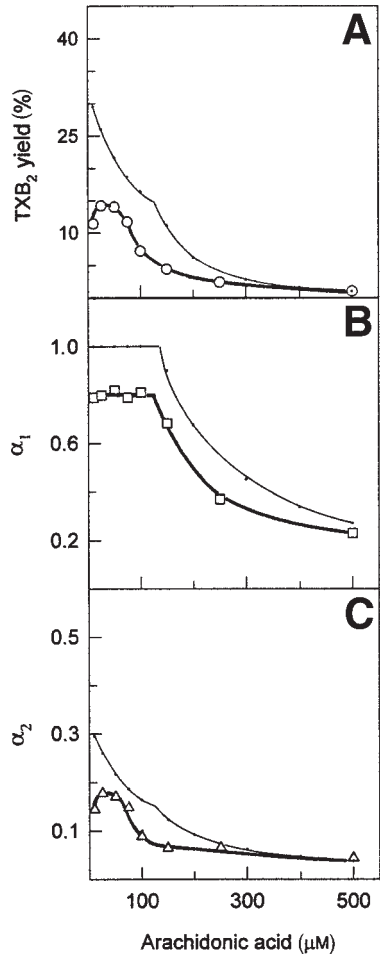


Fig. 6. Effect of arachidonic acid concentration on TXB_2 yield **(A)** and efficiencies of the PG synthase **(B)** and TX synthase **(C)** reactions. PG synthase, 0.12 U/mL; TX synthase, 8 mU/mL. (—○—; —□—; —△—), Experimental data; (——), calculated data.

$$\begin{aligned} [\mathrm{EPA}]_0 &<< 3 \cdot K_M^{\mathrm{II}} \\ [\mathrm{PgS}]_0 &\geq [\mathrm{EPA}]_0 / \nu^{\mathrm{I}} \\ [\mathrm{TxS}]_0 &>> (k_{\mathrm{ne}}/k^{\mathrm{II}}) \cdot K_M^{\mathrm{II}} \end{aligned} \tag{6}$$

Determination of Conditions for Minimum Enzyme Consumption

An approach applied for the two-enzyme PGE synthesis in ref. *30* was used. The dependence of the TX yield on the reaction mixture volume was determined under the conditions of constant quantities of all the system components: EPA, PG synthase, and TX synthase. Figure 7 shows that with a decrease in the reaction mixture volume, the product yield slightly increased. When the reaction mixture volume decreased from 15 to 1 L, TX yield increased only by 6%. The product yield was also affected by the quantity of TX synthase in the system. Analysis of the obtained results

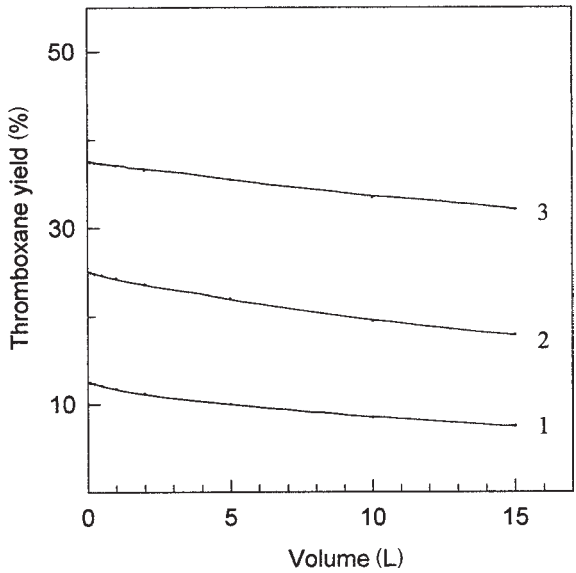


Fig. 7. Calculated data on the effect of the reaction mixture volume on TX yield at constant quantities of enzymes and EPA. EPA, 1 mmol; PG synthase, 1333 U; TX synthase, 33 U (1), 100 U (2), and 300 U (3).

permitted us to determine conditions for the minimum consumption of enzymatic preparations:

$$\begin{aligned} [\mathrm{EPA}]_0 &>> 3 \cdot K_M^{\mathrm{II}} \\ [\mathrm{PgS}]_0 &= [\mathrm{EPA}]_0 / v^{\mathrm{I}} \\ [\mathrm{TxS}]_0 &<< [\mathrm{EPA}]_0 / v^{\mathrm{II}} \end{aligned} \qquad (7)$$

Effect of Separate Addition of Enzymes on TX Yield

As already mentioned, TX synthesis can be influenced by TX synthase inhibition by EPAs and by sorption of the latter on ballast proteins. We hypothesized that the role of these processes on TX synthesis could be reduced by the introduction of TX synthase into the reaction mixture some time after the start of the reaction. Truly, this modification of the reaction protocol considerably increased the product yield. We determined the dependence of TX yield on the time of EPA incubation with PG synthase after which TX synthase was added. The maximum yield corresponded to 1 to 2 min, i.e., the time of virtually complete EPA conversion to PGH through the PG synthase reaction. The observed increase of the TX yield with the separate addition of the enzymes depended on the initial TX synthase concentration (Fig. 8) and was practically independent of EPA concentration (data not shown). At the TX synthase concentration of about 20 mg/mL, TX yield increased almost twofold. This effect can be accounted for by a reduction of EPA sorption on ballast proteins from the TX synthase preparation.

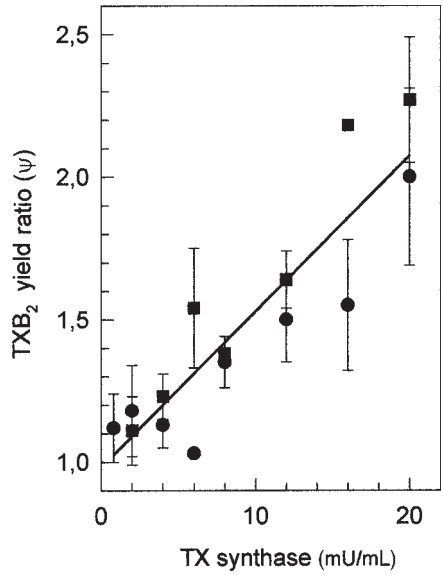


Fig. 8. Effect of separate addition of enzymes on TX synthesis. ψ, ratio between TXB_2 yields at TX synthase addition in 1 min after the reaction start and at simultaneous addition of the enzymes. (—●—), TXB_2; (—■—), TXB_3.

Table 2
Effect of Reducing Agents on 12-HETE Synthesis

Reducing agent	Concentration (μ*M*)	Degree of arachidonic acid conversion (%)	12-HETE yield (%)
None	—	56	9
L-Adrenaline	10	55	9
	50	45	12
	500	61	43
Reduced glutathione	10	52	10
	50	49	15
	500	65	46

Optimization of 12-HETE Synthesis

Microsomal 12-LO from human blood platelets as well as PG synthase or TX synthase is irreversibly inactivated during the reaction (data not shown). This process can be described by Eq. 5. Thus, the maximum product yield at the minimum enzyme consumption is reached at the initial 12-LO concentration equal to $[EPA]_0/\nu^{LO}$ (in which ν^{LO} is the number of 12-LO turnovers before its complete inactivation).

It was found that the addition of some reducing agents to the reaction mixture considerably increased 12-HETE yield (Table 2). The strongest effect was observed in the presence of L-adrenaline and reduced glu-

Table 3
Estimation of Productivity of TX and 12-HETE Synthesis

	Product yield[a]		
End product	%	SVG (mg/kg)	HPM (mg/kg)
TXB_2	14 (50)[b]	431 (1300)	25 (67)
TXB_3	6	54	22
12-HETE	45 (100)	—	62 (125)

[a]HPM, human platelet mass.
[b]Calculated data are given in parentheses.

tathione, in which case the formation of byproducts was substantially reduced. In parallel, the degree of arachidonic acid conversion was practically not changed. This indicates that the observed effect was not related to stimulation of the lipoxygenase reaction.

Evaluation of Efficiency of TX and 12-HETE Enzymatic Synthesis

Table 3 shows the results of TXB_2 and 12-HETE syntheses from arachidonic acid and TXB_3 synthesis from thymnodonic acid that were run under conditions determined by Eq. (7). We also made calculations of the maximum theoretical yields of TX and 12-HETE. According to conditions determined by Eq. (7), TX yield depends on the number of PG and TX synthases turnovers before their complete inactivation as well as on the content of these enzymes in SVGs and human blood platelets, respectively. Calculated with the experimental values of these parameters, theoretical values for TX yield are presented in Table 3. Calculated and experimental data demonstrate that the TX synthesis from thymnodonic acid requires several times more of the SVGs but almost the same amount of the human blood platelets as compared with TX synthesis from arachidonic acid.

Discussion

Human blood platelet microsomes were used for TX and 12-HETE syntheses from EPAs. The microsomes are known *(1)* to contain both cyclooxygenase (PG and TX synthases) and lipoxygenase systems of EPA metabolism. The initial source of the microsomes was the platelet mass obtained as a byproduct of isolation of some proteins from fresh blood plasma. In Moscow, the production rate of these proteins yields up to a few kilograms of the platelet mass per year.

Incubation of tritium-labeled arachidonic acid with human blood platelet microsomes led to formation of the three major products: TXB_2, HHT, and 12-HETE (Fig. 1). Their ratio depended on the concentration of microsomes (Fig. 2). An increase in the concentration of microsomes decreased the fraction of cyclooxygenase products including TX. One of the possible explanations for this result is that 12-HpETE formed in the

lipoxygenase reaction inactivates TX synthesis enzymes. It has previously been shown that 15-HpETE has the same effect on PG synthase *(41)* and TX synthase *(15,18)*.

Besides TXB, during the TX synthase reaction, HHT and malonic dialdehyde are formed from arachidonic acid and thymnodonic acid. They can also be formed via nonenzymatic fragmentation of PG endoperoxide *(42)*. The TXB/HHT ratio was 1:1 when the enzyme from human blood platelets was used *(10–12)* and 1:2 with the enzyme from pig lungs *(15)*. Our results demonstrate that this ratio depends on the reaction conditions. Under the conditions of the maximum TXB yield, the TXB/HHT ratio was 1:1.4 (Fig. 1). This means that when unpurified enzymatic preparations are used, HHT can also be formed as a result of a nonspecific conversion of PG-endoperoxide by ballast proteins.

Blood platelets and crude preparations derived from them are known to contain a high peroxidase activity *(25)*, and, hence, 12-HpETE formed in the lipoxygenase reaction is rapidly reduced to 12-HETE. The same effect was observed when microsomes were used as enzymatic preparations (Fig. 1). Some PG synthase inhibitors are also capable of inhibiting the peroxidase activity of blood platelets *(38)*. We demonstrated that in the reaction of arachidonic acid conversion by human blood platelet microsomes, indomethacin is an efficient inhibitor of blood platelet peroxidase.

We have previously shown that the two-enzyme PGE synthesis is limited by a number of processes involving PG synthase inactivation during the reaction, nonenzymatic destruction of the intermediate PG endoperoxide, inhibition of the PGE synthase by EPAs, and others *(30)*. All these processes also take place in the TX synthesis.

PG synthase from human blood platelets is known to be irreversibly inhibited in the reaction like the enzyme from SVGs *(28)*. In addition, the second enzyme of the TX synthesis, TX synthase, is also subject to inactivation during the reaction *(15–18)*. This inactivation takes place in the reaction with both the isolated enzyme *(15,17)* and the native blood platelets *(17)*. Supposedly, TX synthase inactivation can play an important role in the regulation of thrombogenesis *(17,18)*. Nevertheless, the molecular mechanism of this process is not clear.

The kinetics of the inactivation of TX and PG synthases is described similarly (Eq. 5) and is usually referred to as mechanism-based inactivation (reviewed in ref. *43*). A detailed kinetic analysis of such reactions has previously been given *(19)*. In our case, the enzyme inactivation is characterized by the number of enzyme turnovers before its complete inactivation (Table 1). We showed that the highest effect of the inactivation of TX synthase on the product yield takes place in excess of EPA (Fig. 3C).

Partially purified PG and TX synthases were used to determine conditions for the maximum product yield (Figs. 4–6). A mathematical model of the two-enzyme TX synthesis helped to present it in a generalized form (Eq. 6). Obviously, under these conditions, the effects of the factors limiting TX synthesis is minimal. However, for practical applications of this method

of TX synthesis, it was necessary to determine the conditions of the minimum consumption of enzymes in the reaction. Our studies demonstrated that the yield of the end product calculated with respect to the consumption of enzymes depends little on the concentrations of the system components but does have a strong dependence on their ratio (Fig. 7). This means that the product yield is not actually influenced by the volume of the synthesis. Practically, the synthesis is more convenient to run in a small volume.

Our studies demonstrated that thymnodonic acid is a less preferable substrate compared to arachidonic acid (Fig. 5A,B). The amount of PG synthase to reach the maximum product yield was larger in the case of thymnodonic acid. Similar results were obtained in the studies of the conversion of the exogenous EPAs by human blood platelets *(44)*. A comparison of the kinetic parameters of the PG and TX synthase reactions for corresponding substrates explains these results: TX synthase reactions of PGH_2 and PGH_3 conversions have close values of kinetic parameters *(35)*, whereas for the PG synthase reaction, kinetic parameters of arachidonic acid and thymnodonic acid conversion are different *(30)*. With thymnodonic acid as a substrate, PG synthase can make 10 times fewer turnovers before its complete inactivation than with arachidonic acid.

The conducted studies showed that the TX yield can also be considerably reduced by sorption of EPAs on proteins and nonspecific PG endoperoxide conversion by ballast proteins. We demonstrated that the effect of sorption of EPAs on TX synthesis can be largely prevented by the separate addition of PG and TX synthases (Fig. 8). In this case, TX yield increased 1.5- to 2-fold. The optimum time for the TX synthase addition after the reaction start corresponded to the time for a virtually complete EPA conversion to PGH.

Our results conclude that the major factors affecting the consumption of TX synthase are the inactivation of the enzyme during the reaction and the nonenzymatic destruction of the intermediate PG endoperoxide. Unfortunately, there are no published data on the influence of any factors whatsoever on the TX synthase inactivation. It is known that the inactivation also takes place in the reaction with the immobilized enzyme *(16)*.

12-LO from human blood platelet microsomes can also be inactivated in the reaction. Kinetically, 12-LO is inactivated in a way similar to the other enzymes of eicosanoid synthesis including PG and TX synthases. Thus, in 12-HETE and 12-HpETE syntheses, the enzyme inactivation in the reaction is also one of the major limiting factors.

12-HETE synthesis in the presence of reducing agents considerably increased the product yield. This was owing not to a larger degree of arachidonic acid conversion, but to a less effective formation of byproducts that can be formed by both enzymatic and nonenzymatic 12-HpETE conversion.

It is known that TX synthase and 12-LO isolated from various sources have similar catalytic characteristics *(15,23)*. Hence, our results can be used in future work with the corresponding enzymes from other sources.

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