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Effects of Arachidonic Acid Concentration on Prostaglandin Biosynthesis and Feasibility of Semibatch Processes

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ABSTRACT

The reaction characteristics of prostaglandin E_2 biosynthesis by PGH-synthase and PGE_2 isomerase and the substrate dependency of this biosynthesis were studied. The activity of PG-synthases was blocked by the inhibitory action of one or more byproducts, probably resulting from the action of PGH-synthase. This inhibitory action then appeared to be partly reversible, indicating that the substrate and the inhibitor compete for the catalytic sites. According to these findings, the feasibility of a successful semibatch biosynthesis was investigated. A combination of the substrate concentration reducing procedure and the semibatch process resulted in an about 3.5-fold higher increase in the total amount of PGE_2 formed in comparison with the batch results obtained at the substrate concentration of 1.0 mg/cm^3. Since the cost of enzyme is a governing factor in this biosynthesis, development of semibatch biosynthesis of PGE_2 becomes a matter of economic importance.

Index Entries: Prostaglandin; arachidonic acid; biosynthesis; liposome; semibatch reaction.

INTRODUCTION

The prostaglandin synthase activity associated with the microsomal fraction of most mammalian cells catalyzes the conversion of certain polyunsaturated fatty acids (C_{20}) into a wide array of prostaglandins that

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possess a wide spectrum of biological activity (*1,2*). Since their low concentrations in tissues and also multiple asymmetric carbon centers make the cost of their chemical synthesis or extraction prohibitively expensive, considerable interest has been aroused on the feasibility of their commercial production by means of fermentative and enzyme engineering processes. In this experimental work, the biosynthesis of prostaglandin E_2 (PGE_2) from arachidonic acid (AA, C_{20} unsaturated fatty acid) as the starting material was investigated. In this bioconversion, ram seminal vesicular microsomes (acetone-pentane powder) were used as biocatalysts because of their high activities and also because they are free of cytoplasmic prostaglandin inactivating enzymes.

According to a series of the mechanisms depicted in Fig. 1, the major step in regulation of biosynthesis and metabolism of prostaglandins is the conversion of arachidonic acid to prostaglandin H_2 (PGH_2) by the action of prostaglandin H-synthase. This membrane bound enzyme has been reported to possess two metabolic activities; first, cyclooxygenation in which arachidonic acid converts to PGG_2, and, second, peroxidation in which PGG_2 reduces to PGH_2 (*3*). Endoperoxide E-isomerase then mediates the further metabolism of PGH_2 to PGE_2. This enzyme is a glutathione-dependent enzyme, which also resides in the microsomal acetone-pentane powder.

Since PG-synthase is a membrane-bound enzyme, liposomization (reconstitution of PG-synthase into bilayer membrane) was supposed to improve the reaction properties. In this work, the effect of substrate concentration on prostaglandin synthetic reaction and the results obtained from the study on the feasibility of semibatch biosynthesis are discussed for the cases of both microsome and liposomized microsome.

MATERIALS

Sodium arachidonate (purity: 90%) was purchased from Sigma Chemical Co. Acetone-pentane powders of ram seminal vesicular microsomes were obtained from Funakoshi Pharmaceutical Co. and stored at −50°C. DMPC (D-L-α dimyristoylphosphatidylcholine) was provided from Sigma Chemical Co. All other chemicals used were of reagent grade.

METHODS

Enzymatic Assay

A buffer solution containing 0.0312 *M* of sodium EDTA, 0.0005 *M* of hydroquinone, and 0.0020 *M* of glutathione was adjusted to pH 8.0 by the addition of sodium hydroxide. A 10 mg portion of microsomal acetone-pentane powder was added to 2 cm^3 of the buffer solution containing an

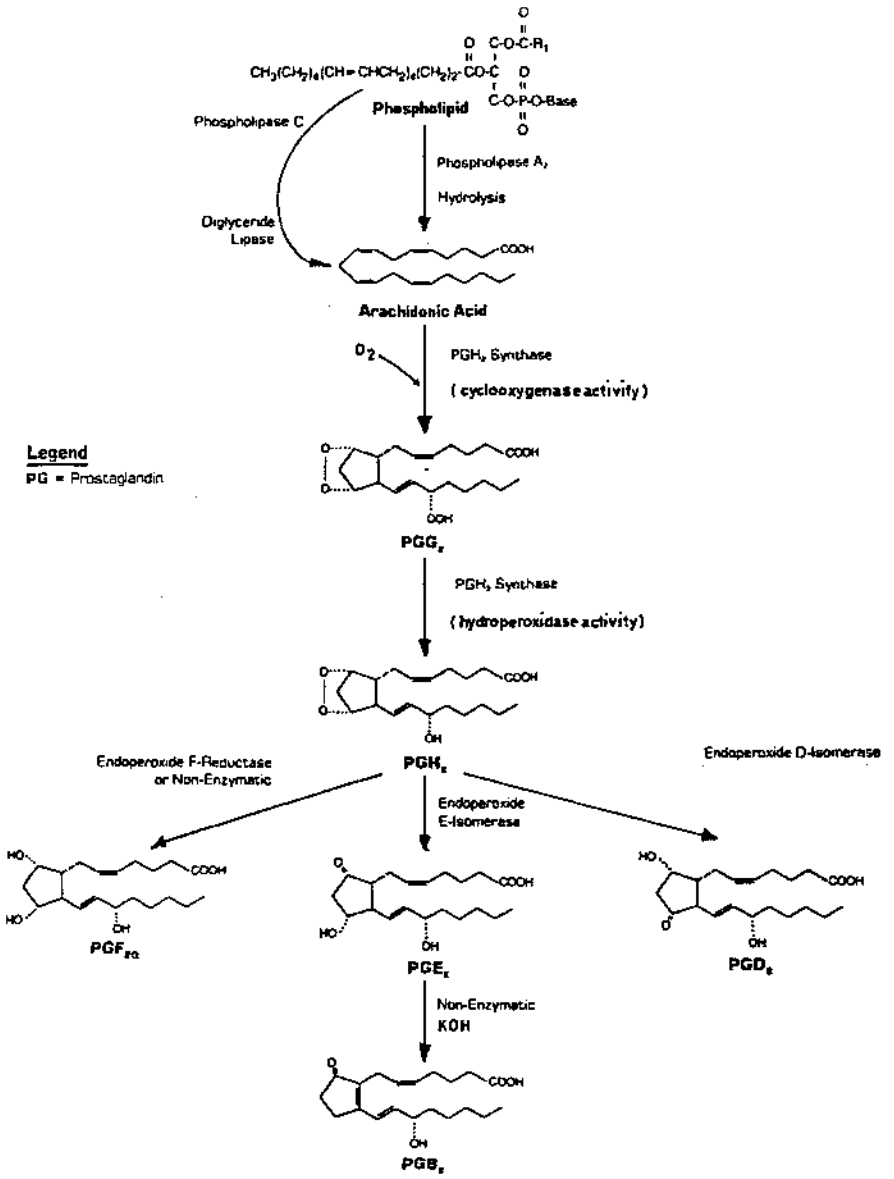

Fig. 1. The metabolism of arachidonic acid to the major prostaglandins PGE_2, $PGF_{2\alpha}$, and PGD_2. Alkaline treatment of PGE_2 to PGB_2 is used for the identification of PGE_2.

appropriate amount of sodium arachidonate. The reaction mixture in a test tube was then incubated in a water-bath at 30°C with agitation by a magnetic stirring bar. At the end of the incubation period, the mixture was acidified by 2 *M* citric acid (about 0.25 cm^3) to pH 2–3. PGE_2 produced in the reaction mixture was extracted three times with 25 cm^3 portions of methylene chloride in a separatory funnel. The solvent was then removed

with a rotary evaporator, and the residue was dissolved into 20 cm^3 of methanol. Two aliquots of the methanolic solution (2 cm^3) were taken, one for the measurement of PGE_2 concentration and the other for the measurement of unreacted arachidonic acid. PGE_2 was converted to prostaglandin B_2 (PGB_2) by the addition of 0.08 cm^3 of 3 *M* KOH (in methanol) per 2 cm^3 of the methanolic solution and further incubation at 50°C for 30 min, according to the metabolic pathway shown in Fig. 1. The concentration of PGB_2 in 2 cm^3 of methanol was measured by a HPLC system (Hitachi 655) with a Wakopak ODS-5 column (4.6 mm × 15 cm) at a wavelength of 278 nm. The solvent system used was 1% aqueous acetic acid: methanol (35:65) with a flowrate of 1.0 cm^3/min.

The concentration of unreacted arachidonic acid was measured by the fluorescent labeling method with use of ADAM (9-anthryldiazomethane) (*4*). For this purpose, the aliquot of the methanolic solution (2 cm^3) taken for the measurement of arachidonic acid, after dilution by half, was added to 0.1 W/V% methanolic ADAM solution in the ratio of 1:4. The resulting mixture was left at room temperature for 60 min. The labeled compounds were measured using a HPLC system (Shimadzu, LC-6A) with a Wakopak ODS-5 (4.6 mm × 15 cm) and a flow through fluorometer (Shimadzu, RF-540). Arachidonic acid was analyzed by a gradient elution from methanol 100 to methanol: water, 75:25 with a flowrate of 1.0 cm^3/min, and the fluorescence was measured at 412 nm and excitation at 365 nm.

Liposomization of Microsomes

A 10 mg portion of acetone-pentane powder was reconstituted into 20 mg of DMPC (T_c = 23°C) using the same method described in the previous report (*5*).

RESULTS AND DISCUSSION

The Reaction Characteristics of Microsomes and Liposomized Microsomes

The effect of arachidonic acid concentration on the time course of PGE_2 synthetic reaction is illustrated in Fig. 2. This figure shows a favorable improvement in PGE_2 yield through the reduction of the substrate concentration. As is obvious, at high concentrations of AA, the reaction proceeded rapidly and approached an asymptote within 15 min, whereas at low concentrations of AA (0.5 or 0.25 mg/cm^3), the reaction prolonged and reached a plateau slowly within 1–2 h. From AA concentration of 0.25 mg/cm^3, any further reduction in the substrate concentration caused less productivity owing to low availability of the substrate to enzyme. These results, and those presented in the previous report (*5*) based on the favorable effect of futher addition of the substrate when the reaction has

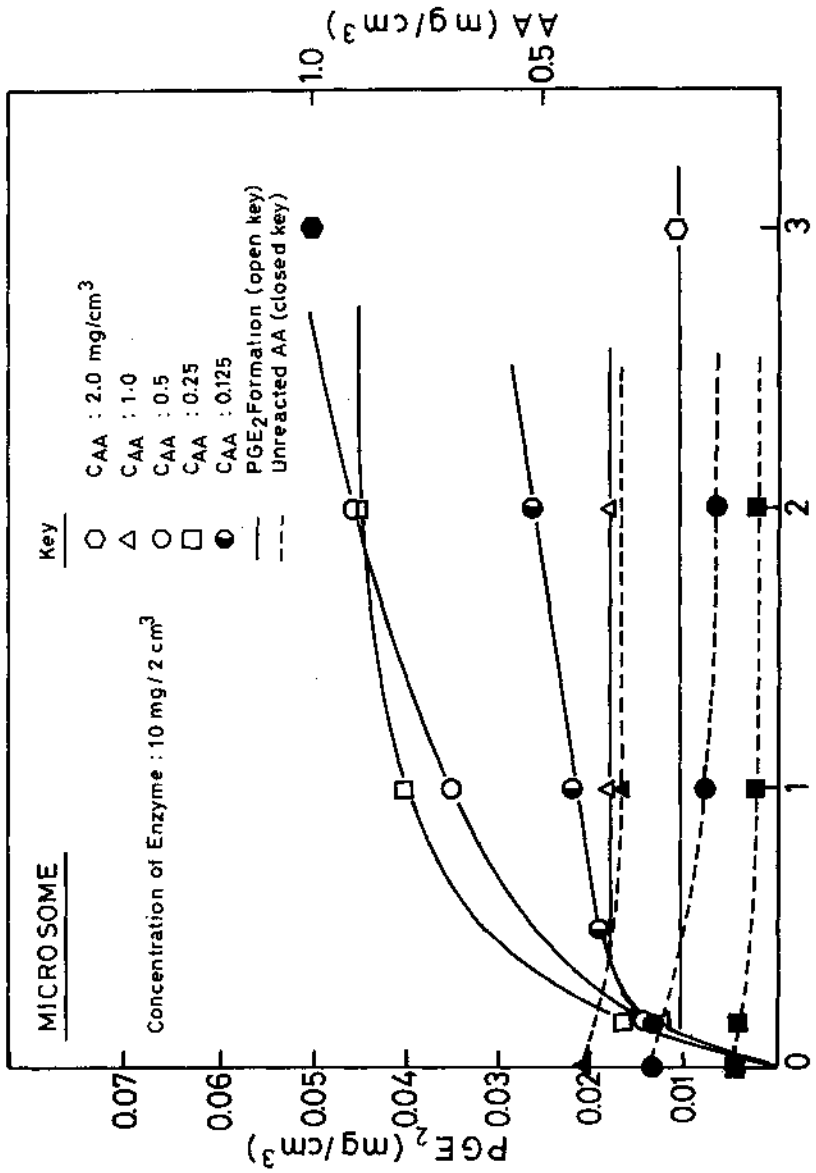

Fig. 2. Time courses of PGE_2 formation and unreacted arachidonic acid at various concentrations of arachidonic acid in the case of microsome.

already plateaued, suggest that the termination of PGE_2 synthetic reaction might be caused by inhibitory action of one or more reaction products (probably substrate analog). This evidence, in agreement with the concept of Wallach et al. (*6*), indicates that the biosynthesis of PGE_2 is accompanied by the synthesis of an inhibitor (or inhibitors), which is strongly substrate-dependent. Although Marnett et al. (*7*) reported the substrate inhibition of PGH-synthase by arachidonic acid, from the measured initial rates of PGE_2 and the times reaching the plateau at varying concentrations of AA, substrate-inhibition seemed to be less effective in comparison with those caused by one or more reaction products. Prostaglandin H synthase, which is responsible for the conversion of arachidonic acid to PGH_2, is supposed to be more sensitive to this inhibitor than endoperoxide E-isoermase, which contributes to the conversion of PGH_2 to PGE_2. The reduction of AA concentration from 1.0 mg/cm^3, almost the optimal concentration at which AA was utilized in Wallach et al.'s work (*6*), to 0.25 mg/cm^3 resulted in an about 2.5-fold higher increase in total amount of PGE_2 formed.

The time courses of unreacted arachidonic acid are also shown in Fig. 2. Despite low conversions, especially at high concentrations of the substrate, only half of the anticipated amount could be detected. Attempts were made to extract arachidonic acid from the reaction mixture in the presence of the enzyme when no reaction occurs. To do this, the reaction mixture containing arachidonic acid was acidified prior to the addition of the enzyme. We found that in the case of microsome, arachidonic acid was only partly extractable by the procedure outlined in Methods. This problem did not appear in the case of liposomized acetone-pentane powder, which will be described later. Since acetone-pentane powders utilized in these experiments are microsomes that have been treated by acetone and pentane, the hydrophobic substrate is adsorbed by these hydrophobic proteins whose lipid contents have previously been reduced in the treatment by the organic reagents. As shown in this figure, AA consumption proceeded and reached a plateau almost at the time when PGE_2 formation terminated.

Figure 3 shows the effect of arachidonic acid concentration on the time course of PGE_2 formation in the case of liposome. Since some membrane bound enzymes require phospholipids that induce favorable conformational changes of either enzyme or substrate and phase transition changes (*8*), we made some investigation on the effect of liposomization of microsomal acetone-pentane powder (*5*). According to Fig. 3, the reaction was enhanced even at high concentrations of arachidonic acid. From the comparison between Figs. 2 and 3, the time courses of PGE_2 at AA concentrations of 1.0 and 0.5 mg/cm^3 in the case of liposome were very similar to those obtained at AA concentrations of 0.5 and 0.25 mg/cm^3 in the case of microsome. One explanation for this phenomenon would be a distribution of arachidonic acid into bilayer vesicles, which causes a

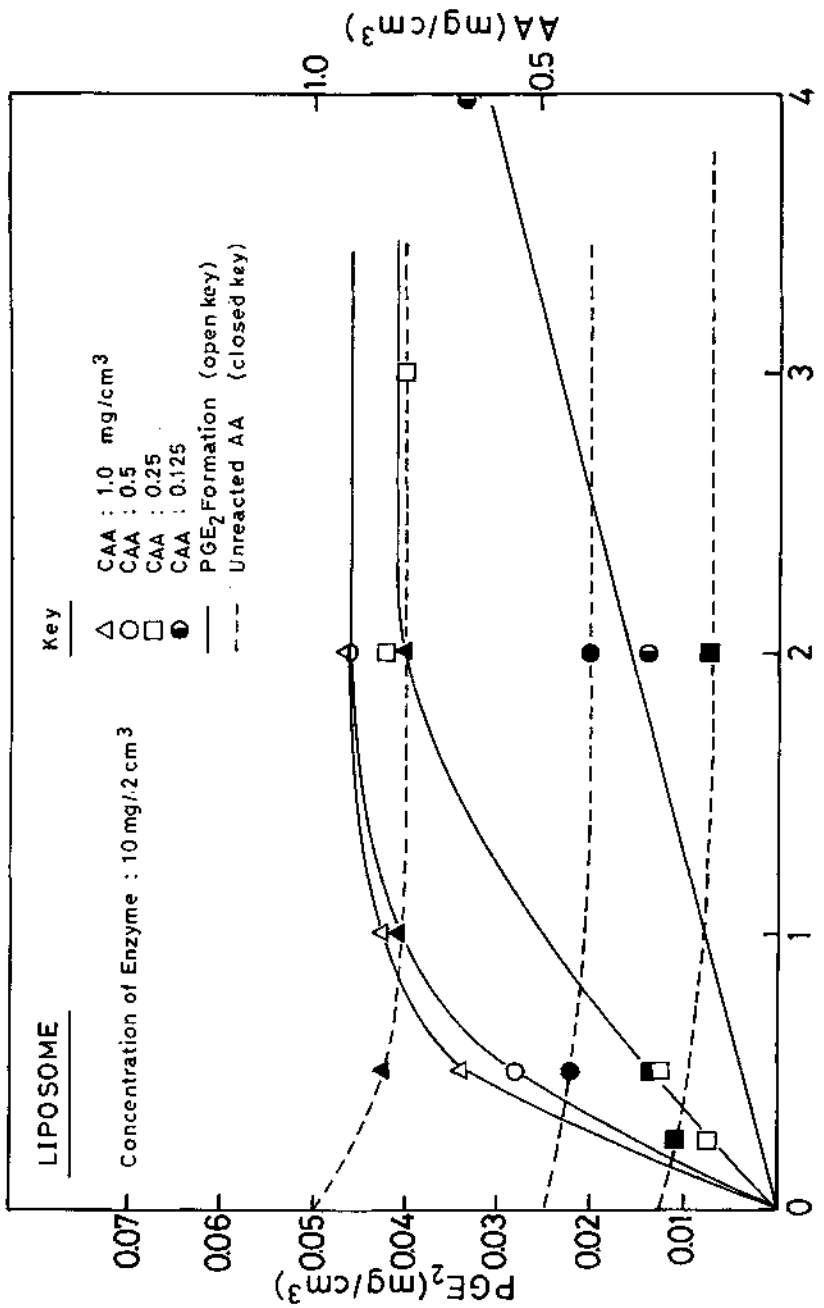

Fig. 3. Time courses of PGE_2 formation and unreacted arachidonic acid at various concentrations of arachidonic acid in the case of liposome.

favorable dilution according to the previous results. Slow velocities at low concentrations of AA are caused by low and slow accessibility of AA to the enzyme owing to the distribution and diffusion into bilayer membrane. From this figure, it is apparent that arachidonic acid has become extractable owing to reconstitution of the hydrophobic proteins and partition of arachidonic acid into bilayer vesicles.

The evidence on the inhibitory action of one or more byproducts suggests that development of an improved method for biosynthesis should involve entrapment of this inhibitor. Hence, some inhibitor-entrapment experiments were run by the addition of vacant liposome (including no enzyme) to the reaction mixture in both cases of microsome and liposome. Figure 4 shows that this treatment with the vacant liposomes has improved the reactivity of PG-synthase and prolonged the prostaglandin synthetic reaction. The fact that the PGE_2 formation in liposome + vacant liposome exceeds all the results shown in Fig. 3 indicates that this high reactivity is not only caused by the distribution of arachidonic acid into vacant liposome, but that the inhibitor seems to be also entrapped. The difference between PGE_2 formation rates in the cases of liposome and microsome + vacant liposome may also suggest the effect of liposomization owing to stability of enzyme and favorable conformational changes of both the enzymes and substrate incorporated into the lipophilic environment. Especially, the specificity of PGH-synthase to attack the CH_3-terminated chain of arachidonic acid owing to it's greater hydrophobicity than COOH-terminated chain *in vivo*, led us to consider the probability of favorable conformational changes of either the enzyme or the substrate embedded in a lipophilic environment.

Efforts were made in the detection of byproducts by applying the fluorescent labeling method (using ADAM). However, besides $PGF_{2\alpha}$, no byproduct with an inhibitory effect could be identified.

The studies on heat-lability of acetone-pentane powder were achieved by preincubations of the enzymes prior to addition of the substrate. For 1-or 2-h incubations at 30°C, no appreciable inactivation owing to heat instability was observed. These results are presented in Table 1. These data also suggest that the termination of the PGE_2 synthetic reaction is not caused by the release of one or more fatty acids associated with microsome according to the actions of phospholipases.

According to the observations made by Ham et al. (*9*) and Lands et al. (*10*), oxidizing agent [O_x] released during the enzymatic reduction of PGG_2 to PGH_2 was shown to be destructive toward the component of the PG-synthase system. The negation of this effect was accomplished by the use of phenol (*10*) and other radical scavengers such as α-tocopherol and superoxide dismutase, but no improvement through the addition of these agents was observed in the present work. In this case, hydroquinone, which is present in the reaction mixture with an action of antioxidant, is capable of prevention of this destructive effect to some extent.

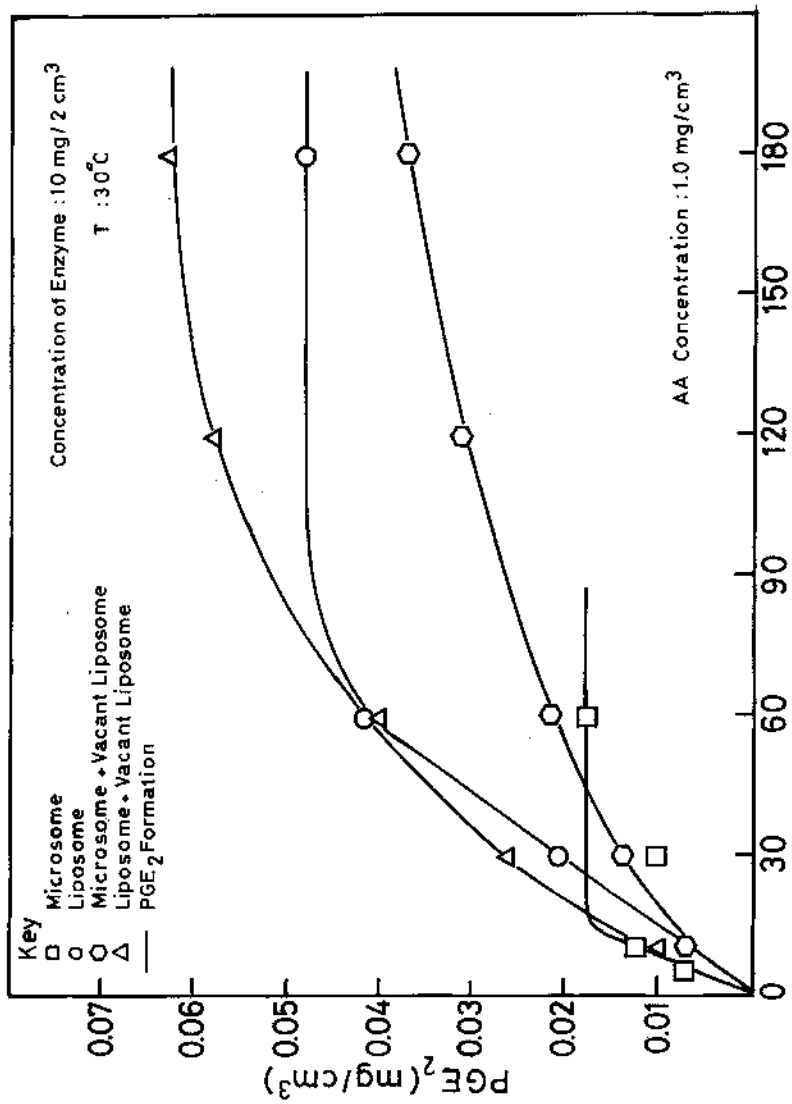

Fig. 4. Effect of addition of vacant liposome on entrapment of the inhibitor in both cases of microsome and liposome.

Table 1
Heat Stability of Acetone-Pentane Powder
in the Cases of Microsome and Liposome

Utilized form of PG-synthase	Preincubation time (h), T=30°C	Reaction time (h), T=30° C	PGE_2, mg/cm³
Microsome C_{AA}:0.5 mg/cm³	0	2	0.055
	1	2	0.055
	2	2	0.060
Liposome C_{AA}:1.0 mg/cm³	0	1	0.0425
	1	1	0.044
	2	1	0.0415

Biosynthesis of PGE_2 in Semibatch Processes

According to the results obtained, at high concentrations of AA the formation of PGE_2 was significantly inhibited by the inhibitor that reversibly competes with the substrate AA. These findings enabled us to investigate the feasibility of semibatch biosynthesis. Thus, the reaction was initiated at low concentration of AA (0.125 mg/cm³) and allowed to go to the plateau point. At this point, further increments of the substrate were added to the reaction mixture. But the addition of arachidonic acid permits only limited synthesis of PGE_2 until the concentration of the inhibitor, which is also being synthesized, increases to the concentration where PG-synthase is inhibited. Therefore, the amount of arachidonic acid added at each step plays an important role in enhancing the reaction in the favor of PGE_2. According to the results graphically demonstrated in Fig. 5, at high concentrations of inhibitor, large additional amounts of AA are required to induce a favorble replacement between AA and the inhibitor. This explanation is consistent with data presented in Fig. 6, which will be described later, as a difference appeared between the time courses of PGE_2 in stepwise and continuous semibatch processes.

In the next step, we considered a continuous pattern in addition of AA into the reaction mixture. In these experiments, the substrate was added to the reaction mixture by using an infusion pump with a flowrate of 0.09 cm³/h. Figure 6 shows the time course of PGE_2 formation in continuous semibatch process with an optimal molar flowrate of AA, comparing with the results obtained in stepwise semibatch process in the case of microsome. From these results, it is apparent that PGE_2 formation in the continuous semibatch process, although higher in comparison with the batch results, proceeded to a plateau after 2 h, whereas PG-synthase was still capable of further synthesis in the stepwise semibatch process. The results of study on the semibatch processes in the case of liposome are illustrated in Fig. 7. As is obvious, liposome showed the same response to

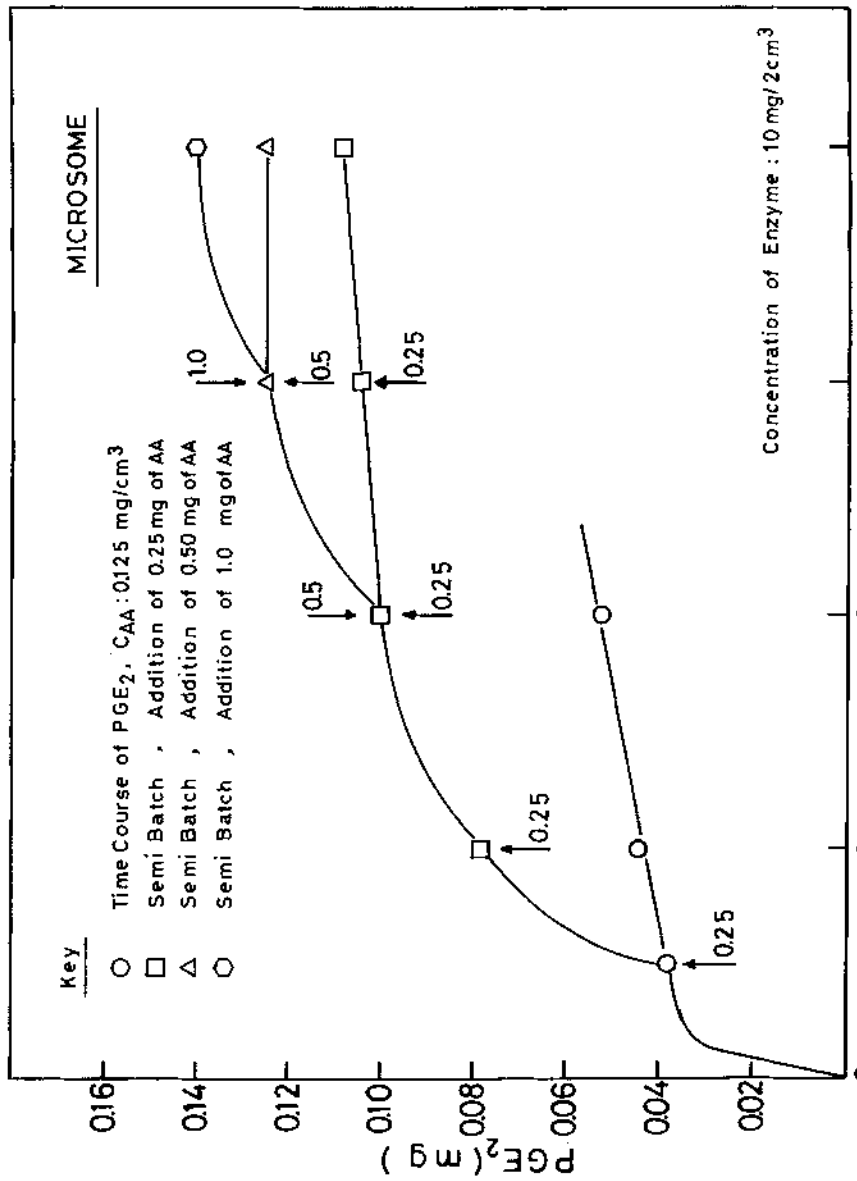

Fig. 5. Effect of further addition of the substrate AA in the case of microsome.

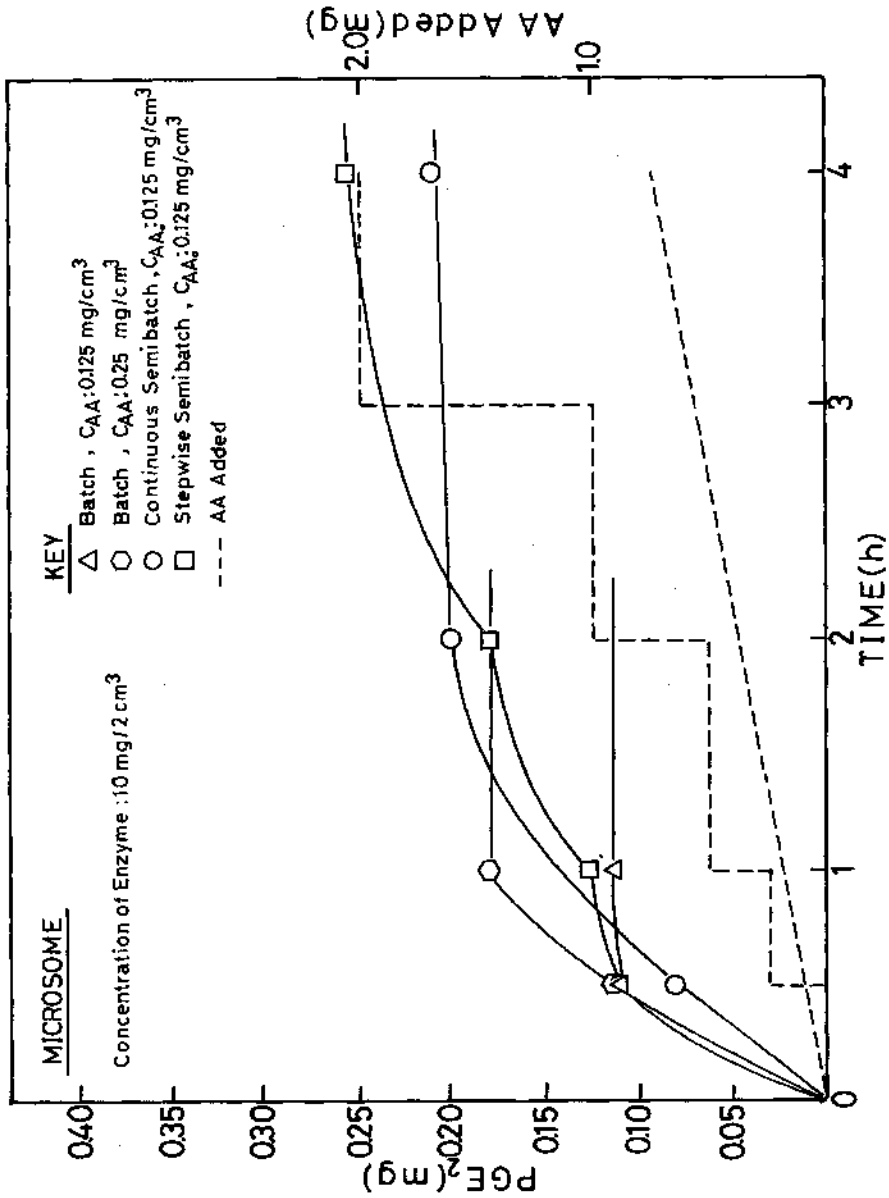

Fig. 6. Time courses of PGE_2 formation in continuous and stepwise semibatch processes in the case of microsome.

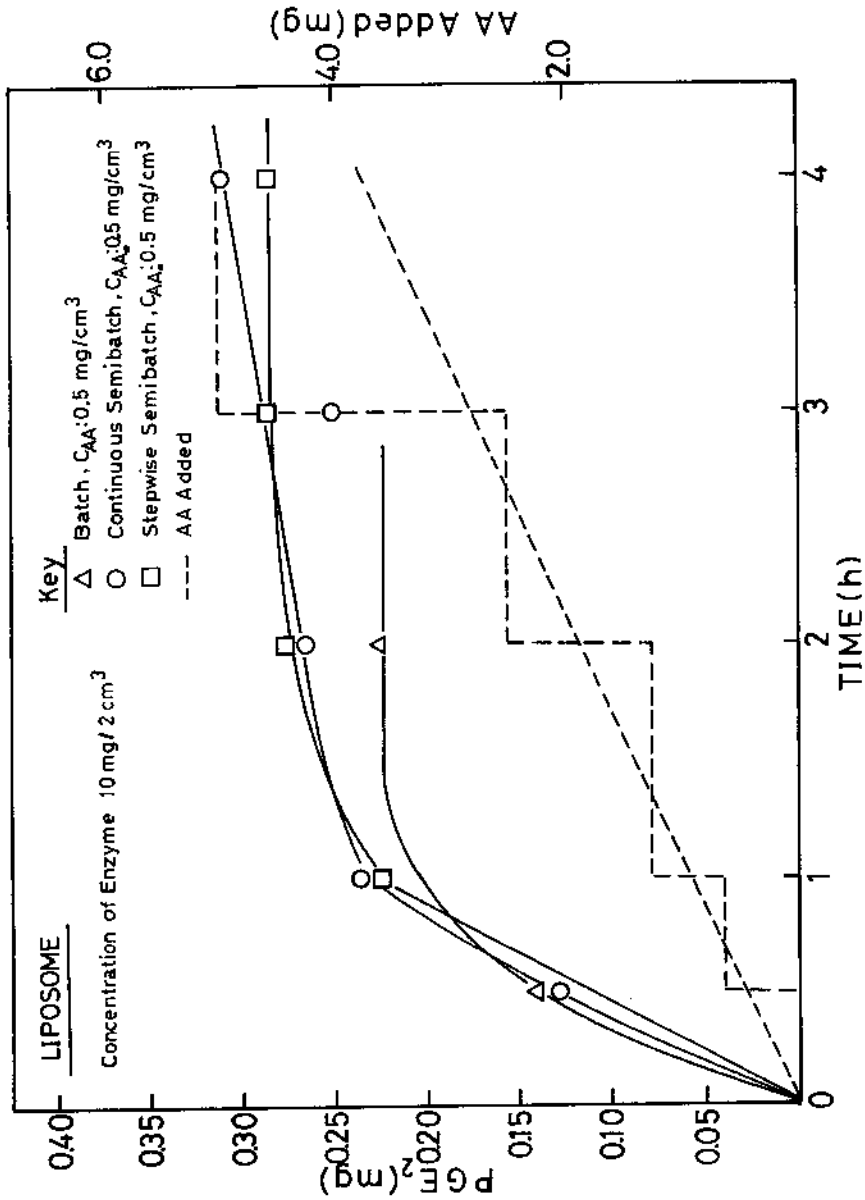

Fig. 7. Time courses of PGE_2 formation in continuous and stepwise semibatch processes in the case of liposome.

either continuous or stepwise addition of the substrate. It is supposed that the distribution of arachidonic acid into bilayer membrane uniforms the effect of different additional patterns of the substrate.

These processes resulted in further 1.4-fold higher increase in the total amount of PGE_2 formed than those obtained in the batch processes for the cases of both microsome and liposome with an optimal initial concentration of arachidonic acid. Since the cost of enzyme is a governing factor in the biosynthesis of prostaglandin E_2, the feasibility of semibatch biosynthesis of PGE_2 becomes a matter of economic importance.

Although the activities of acetone-pentane powders varied through the different lots utilized in this work, the same behaviors of reaction kinetics were observed. The experimental results shown in Figs. 2–4, Fig. 5, and Figs. 6–7 were obtained using the same lots, respectively.

Some investigations were made in the immobilization of PG-synthase and its application in continuous processes. Although we obtained good results in batch studies of immobilized PG-synthase on urethane prepolymers, the continuous investigations were not successful. It seems that any efforts in immobilization of PG-synthase on a suitable carrier, through which both AA and PGE_2 can diffuse, will face the problem of the accumulation of the inhibitor that brings the reaction to end.

To gain a better understanding about the inhibitory actions and those factors that modulate the PG-synthase activity, a comprehensive investigation would be required. This can be achieved by the purification of each enzyme that contributes in this biosynthetic pathway, and thus provides a framework for our further studies.

CONCLUSION

Although there are a number of known and unknown factors contributing to the termination of PG-biosynthesis, this work shows that the activity forming PGE_2 is blocked by the inhibitory action of one or more reaction byproducts. The substrate-dependency of PGE_2 biosynthesis and the reversibility of the inhibitory action led us to develop successful semibatch processes. Since there is still no detailed information about the inhibitory mechanism, our future research would be directed toward a precise investigation of the reaction mechanism and a mathematical model to accommodate our experimental results, with the objective of developing an effective biosynthetic process.

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