Plant-Derived Catalysts and Precursors for use in Prostaglandin Synthesis

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ABSTRACT

Arachidonic acid derivatives such as prostaglandins, thromboxanes, leukotrienes and prostacyclin have been recognized to have significant pharmaceutical potential. Presently they are not available commercially in large amounts because their low concentrations in living tissues and multiple asymmetric carbon centers make the cost of their extraction or chemical synthesis prohibitively expensive. However, the theoretical feasibility of the commercial production of such C-20 compounds by means of fermentative and enzyme engineering processes that bypass animal sources entirely has been demonstrated. The precursor, arachidonic acid, is present in microorganisms adaptable to large-scale fermentation. For the purpose of this study, a model describing changes in arachidonic acid production by the red alga, *Porphyridium cruentum*, in response to induced lipogenesis and variation of light intensity and temperature was developed. In addition, the demonstration that immobilized microsomes containing prostaglandin synthetase activity in a stable amphiphilic gel eliminates the need for the expensive and time-consuming enzyme recovery methods used previously. Furthermore, the ability of a leguminous enzyme to catalyze the synthesis of prostaglandin indicates that low cost catalysts and precursors of plant origin are available for the synthesis of such compounds.

INTRODUCTION

This paper concerns the recent development of a new strategy for the production of arachidonic acid and its pharmacologically active derivatives. Arachidonic acid (5,8,11,14-eicosatetraenoic acid) is an expensive, essential dietary constituent for man. In addition, it is the natural precursor of a large family of structurally related C-20 compounds that include the prostaglandins, thromboxanes, leukotrienes, and prostacyclins, all of which are potent biological regulators (Fig. 1). Among the practical applications of prostaglandins are the treatment of high blood pressure, asthma, ulcers and menstrual cramps, the induction of labor and abortion (1), the promotion of the healing of bone fractures (2), and the synchronization of estrus for the breeding of animals, to name a few (3).

The typical source of arachidonic acid and its C-20 derivatives has been animal tissue (Table I). New sources must be found as the demand for this family of compounds increases in clinical research and pharmacy since, unlike hormones which are produced by specific glands, these arachidonic acid derivatives are synthesized and released in sparingly low concentrations by many tissues and organs and therefore are not concentrated to any large degree in a particular tissue. It was this stumbling block that resulted in their relatively late discovery and characterization, and which has impeded their commercial use. To overcome these shortcomings, the following production strategies shown in Scheme I must be evaluated.

TABLE I

Animal Sources of Arachidonic Acid and its C-20 Derivative Prostaglandin E

Traditional Sources of Arachidonic Acid	
Mammalian	
Testes	
Adrenal Gland	
Liver	
Fat	
Sources of Prostaglandins (4)	**Prostaglandin E (μg/g)**
Vertebrates (Seminal Fluid)	< 40
Prochordates	< 0.01
Arthropods	< 0.1
Mollusks	< 0.1
Coelenterates	< 0.01

Much research has concentrated on the chemical synthesis of the various arachidonic acid derivatives, but it is difficult to avoid using sophisticated and expensive reagents, some of which are quite toxic and difficult to handle even at pilot-plant scale. In addition, racemic product mixtures must be resolved in order to yield pure enantiomeric products. The problem of resolution is especially difficult in the case of C-20 derivatives of arachidonic acid, because they can contain as many as 5 assymetric carbons. Furthermore, they are subject to autoxidation and intolerant of harsh reaction conditions. Such limitations have made their total chemical synthesis prohibitively expensive (5).

Biochemical pathways are of more practical interest since optically active compounds are produced without the

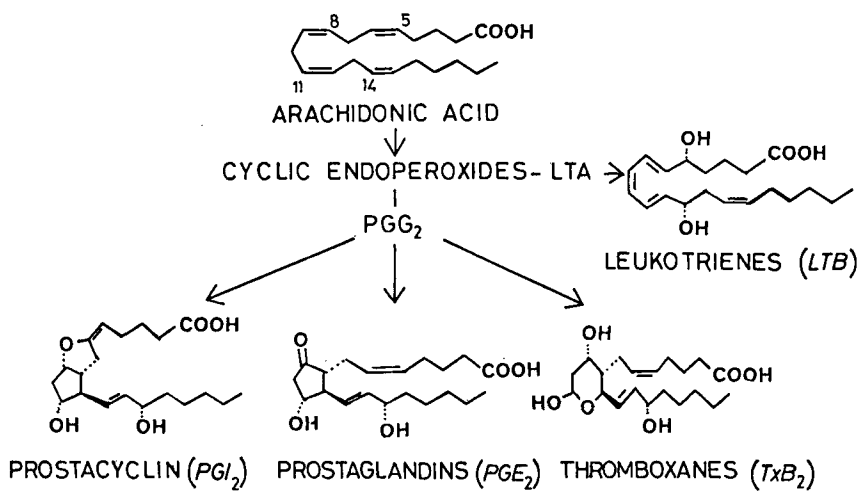
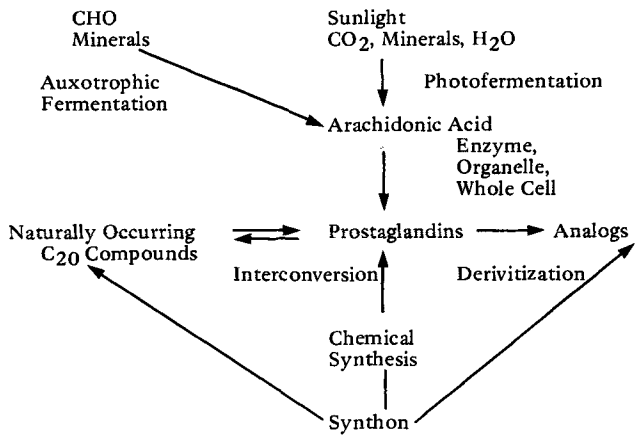


FIG. 1. The metabolism of arachidonic acid to pharmacologically active C_{20} compounds. The abbreviation of the example of each class is given in parentheses.



SCHEME 1. Synthetic Pathways to the Pharmacologically Active C_{20} Compounds

need for chemical resolution. Unlike the synthetic pathways, only a few transformations separate arachidonic acid from prostaglandins (Fig. 2). The first enzymatically catalyzed step is a site-directed double peroxidation and ring-closure, resulting in a prostanoate derivative, PGG, which is transformed rapidly to the major prostaglandins via reductases.

The realization of the large-scale biosynthesis of the C-20 compounds requires that an inexpensive source of arachidonic acid be available, inexpensive biocatalysts be found, and a system that incorporates them to yield desired product be developed.

In Table II, the more promising alternative sources of arachidonic acid are compared to liver and adrenal gland. Japanese patents describe production of arachidonic acid from several microorganisms including *Penicillium* (6). Yet another group, the algae, appeared to be particularly promising because fatty acids can account for as much as 22% of their dry weight (7-10), whereas fatty acids have been reported to account for no more than 9% of the dry weight in the heterotrophic microorganisms examined despite efforts at enhancement (6,11). In addition, the C-20 fatty acids in those organisms naturally comprise less than 2.5% of the total fatty acid content, whereas arachidonic acid alone constitutes as much as 36% in the alga *Porphyridium cruentum* (12).

ARACHIDONIC ACID SYNTHESIS BY *PORPHYRIDIUM CRUENTUM*

The Effect of Temperature

To test the suitability of this single-celled alga for arachidonic acid production, its response to various regimes of light intensity and temperature were investigated (13). Models for both cell growth and arachidonic acid yields were developed. It was found that arachidonic acid yields

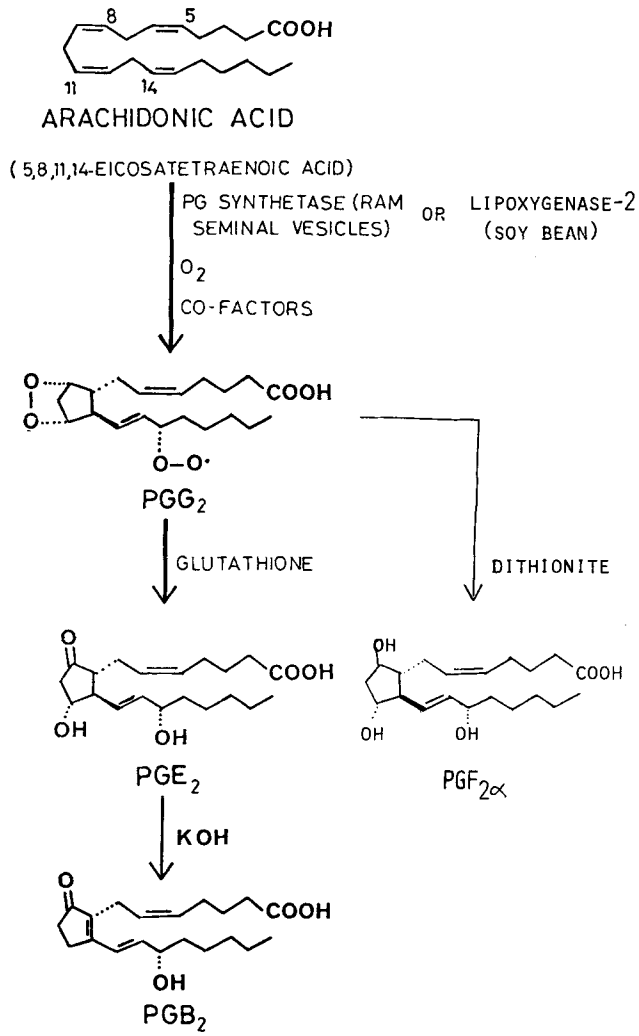


FIG. 2. The conversion of arachidonic acid to the major prostaglandins PGE_2 and PGF_2. Alkaline treatment of PGE_2 to form PGB_2 is useful for spectrophotometric analysis.

per cell were dependent upon the temperature at which the culture was grown, and were highest at low temperatures and low cell concentrations (Fig. 3). In addition, the weight ratio of the two most abundant fatty acids, arachidonic and palmitic, increased with decreasing temperature, from 1.07 for cultures grown at 23-26 C to 1.53 for cultures grown at 18 C. This increase in the amount of a highly unsaturated fatty acid relative to the most abundant saturated fatty acid at low temperature indicates that differences in the viscosities of the fatty acids are exploited by *P. cruentum* to control the viscosities of cellular membranes in response to changes in temperature. That this can be done by adjusting

TABLE II

Novel Sources of Arachidonic Acid

Source	Examples	Fatty Acid (% Dry wt.)	$C_{20:n}$ (% Total FA)	Potential Arachidonic Acid Yields (% Dry wt.)
Animal	Liver	6.5	2–8	0.5
Microorganisms				
Auxotroph	Penicillium	9	3–11	1.0
Autotroph	Algae	3–22	5–36	8.0

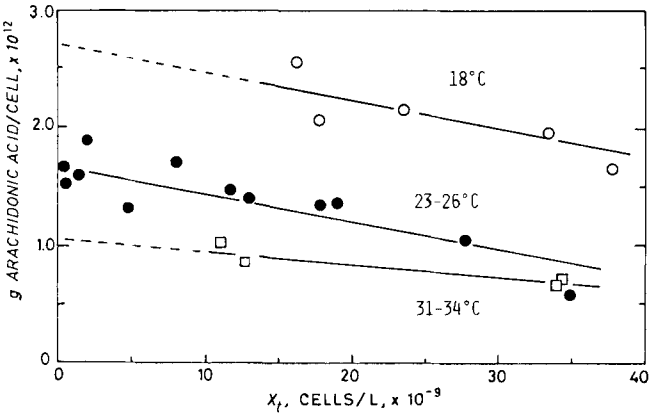


FIG. 3. The effect of temperature and cell concentration on the arachidonic acid yield from the unicellular alga, *Porphyridium cruentum*. ○ - ○, 18 C; ● - ●, 23-26 C; and □ - □, 31-34 C. The light intensity was 8000 lux. See ref. (13) for details.

the proportion of unsaturated and saturated fatty acids within the cell is corroborated by the demonstration (14) that membrane microviscosities in microorganisms are regulated in response to temperature by the means observed in *P. cruentum*. Theories which ascribe to arachidonic acid a unique but unspecified role in algal membrane structure must take these findings into account.

With regard to this study, however, it must be noted that although lowering the temperature of cultivation significantly increased the arachidonic acid yield per cell, it did not result in increased arachidonic acid production per unit time and volume. This finding emphasizes the overriding importance of the input of thermal energy to the system compared to the cell's ability to divert energy to the synthesis of fatty acids in response to changes in temperature. A mathematical model of these relationships is described in a recent paper (13). Values for the rate of arachidonic acid production calculated by means of the model are plotted in Figure 4. It can be seen that the maximum arachidonic acid productivity is predicted to occur during the late exponential phase at the highest temperature attempted.

The Effect of Light Intensity

Arachidonic acid does not exist as the free acid in *P. cruentum*. About 47% of the arachidonic acid in that alga is a constituent of lipids that is known to be concentrated mainly in plasmids (12). It has, therefore, been tentatively assigned a role in the photosynthetic organelles of that algae. Since the amount of pigment per cell was reported to decrease with increasing light intensity, it was postulated that the arachidonic acid content of cells also may vary with light intensity. However, it was found that the amount of arachidonic acid produced by the cells is independent both of light-intensity and any light-dependent changes in cell dry weight and fatty acid composition.

Induced Lipogenesis

A third method of enhancement, namely lipogenesis, demonstrated that cells grown in the presence of linoleic acid at low temperatures contained a three-fold excess of arachidonic acid relative to controls.

A strategy for maximizing the rate of synthesis of arachidonic acid by *P. cruentum* thus requires that cultivation be carried out at temperatures of 32 C or higher in well-mixed, aerated sea water in either batch or continuous culture. Such yields may subsequently be enhanced by artificial induction of lipogenesis in holding tanks at lower temperatures. In addition, the finding that arachidonic acid yields

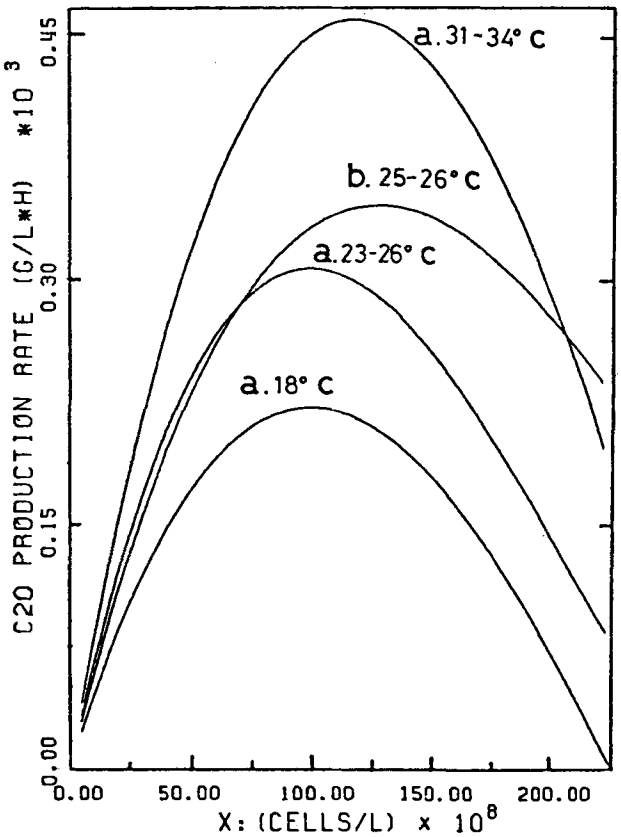


FIG. 4. The effect of cell concentration and temperature on the rate of arachidonic acid production. a) shaking batch culture; b) large-scale batch culture. See ref. (13).

per cell increase with decreasing temperature may be useful for the design of systems minimizing the proliferation of contaminants.

Local conditions, however, may be the ultimate determinant of the feasibility of production by means of autotrophs. Such factors include the availability of warm sea water and CO_2, and the variation and intensity of incident sunlight vs. the price of energy, traditional feedstocks, etc. Recent estimates (15) of the production cost of "clean algal cultures", i.e., single-species algal cultures relatively free of contamination by other organisms, range from $1.50 to $10/kg dry weight, depending on the size and type of culture technique employed. Based on these figures, the cost of producing the red alga, *P. cruentum*, for the purpose of arachidonic acid extraction ranges from $0.15 to $1.00/g of arachidonic acid.

BIOTRANSFORMATION OF ARACHIDONIC ACID TO PROSTAGLANDINS

Given that an inexpensive and expandable supply of starting material is available, does there exist a process by which it can be converted to C-20 compounds of pharmacological interest? The enzymes used to catalyze these processes have been isolated from animal sources such as beef and ram seminal vesicles, as well as rabbit kidneys. In addition, it has been reported (16) that lipoxygenase-2 isolated from soybeans converted arachidonic acid to the doubly peroxidized prostanoate derivative PGG. Treatment with dithionite rapidly converted this intermediate to the prostaglandin PGF_2 with a yield of about 20% of the original arachidonic acid. It had been well known that plant-derived lipoxygenases can catalyze double-dioxygenation of arachidonic acid, but this was the first report that enzymes from these

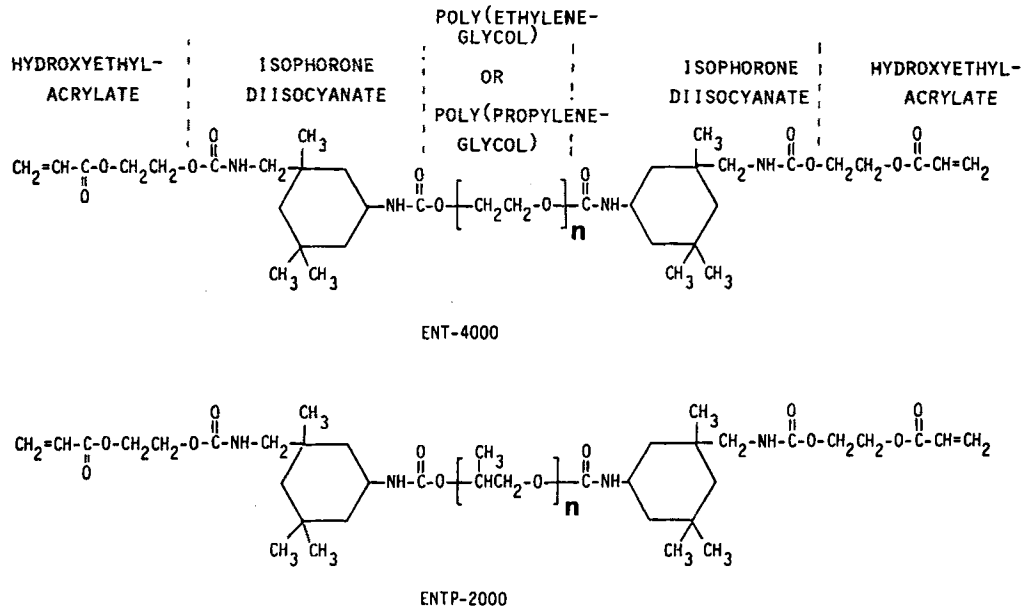


FIG. 5. The structure of photo-crosslinkable prepolymers for the entrapment of microsomes containing prostaglandin synthetase.

sources also can promote the formation of the cyclopentane ring of prostaglandins. The first report of the existence of a prostaglandin in plants, viz., onions, was soon to follow (17).

The discovery of such enzymes in abundant and inexpensive plant sources considerably lowers the cost of a hypothetical prostaglandin production process using arachidonic acid as starting material. Such a system need only involve an enzyme reactor containing an immobilized lipoxygenase or cyclooxygenase, a vessel in which the peroxidated intermediate is reduced to the corresponding hydroxy compounds by means of inexpensive reductants such as dithionite or glutathione, and a column in which the products are purified.

In our preliminary study of the process development at Kyoto University, Professor Sada, Dr. Katoh and I concentrated on immobilization of the enzyme (18). Immobilization of the prostaglandin synthetase enzyme complex in a stable, solid gel support through which both reactants and products can diffuse makes possible batch or continuous synthesis without the need for the expensive and time-consuming enzyme recovery methods used previously, e.g. centrifugation. In this work a photocrosslinkable gel having both lipophilic and hydrophilic properties was assessed as a means of entrapping the prostaglandin synthetase system present in microsomes (Fig. 5). The greater hydrophobicity of ENTP compared to ENT is due to the methyl side chain of its repeating propylene unit. The amphiphilic nature of such gels can be regulated by varying the ratio of the two prepolymers. Their value in the immobilization of enzymes, microbial cells and organelles for the transformation of hydrophobic compounds has been demonstrated by Professor Fukui and coworkers at Kyoto University (19).

Ram seminal microsomes were chosen for the preliminary investigation since they were the most thoroughly studied, commercially available catalyst of the conversion of arachidonic acid to prostaglandins. It was shown that the amount of prostaglandin recoverable from the first cycle of a batch incubation containing immobilized microsomes and arachidonic acid was 35±2% of the PGE formed by an equivalent amount of free microsomes (18). Furthermore, the amount of PGE_2 produced per incubation remained relatively constant during the first four cycles of incubation and decreased linearly during the next five cycles to a level of about 30% of the initial yield per incubation. This gradual decrease in yield probably is due to the instability to heat of prostaglandin synthetase.

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REFERENCES

1. Srivastava, K.C., South African J. Sci. 76:170 (1980).
2. C&E News, Dec. 12 (1983), p. 22.
3. JAOCS 61:476 (1984).
4. Nomura, T., and H. Ogata, Biochim. Biophys. Acta. 431:127 (1976).
5. Bindra, J.S., and R. Bindra, Prostaglandin Synthesis, London, Academic Press, 1977, p. 35.
6. Iizuka, H., T. Ootomo and Y. Kosaka, Japan Kokai Koho 77 64,483 (C1,C12Di/02) (27 May 1977) 4pp.
7. Borointzka, L.J., and A.D. Brown, Arch. Microbiol. 96:37 (1974).
8. Amotz, A.B., and M. Avron, FEBS Lett. 29:153 (1973).
9. Amotz, A.B., and M. Avron, Plant Physiol. 51:875 (1973).
10. Ratledge, C., Enzyme and Microbial Technology 4:58 (1982).
11. Shaw, R., in Advances in Lipid Research, edited by R. Paoletti and D. Kritchevsky, New York, Academic Press, 1966, p. 107.
12. Nichols, B.W., and R.S. Appleby, Phytochemistry 8:1907 (1969).
13. Ahern, T.J.; S. Katoh and E. Sada, Biotechnol. Bioeng. 25:1057 (1983).
14. Hampton, J.J.; R.A. Floyd; J.B. Clark and J.H. Lancaster, Chem. Phys. Lipids 27:177 (1980).
15. Becker, E.W., Process Biochem. 16:10 (1981).
16. Bild, G.S.; S.G. Bhat; C.S. Ramadoss and B. Axelrod, J. Biol. Chem. 253:21 (1978).
17. Attrep, K.A.; W.P. Bellman Sr.; J. Attrep Jr.; J.B. Lee and W.E. Braselton, Lipids 15:292 (1980).
18. Ahern, T.J.; S. Katoh and E. Sada, Biotechnol. Bioeng. 25:881 (1983).
19. Fukui, S.; K. Sonomoto, N. Itoh and A. Tanaka, Biochimie 62:381 (1980).

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