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STUDIES ON THE *IN VITRO* INHIBITION OF PROSTAGLANDIN SYNTHETASE BY FENCLORAC (α,*m*-DICHLORO-*p*-CYCLOHEXYLPHENYLACETIC ACID) AND INDOMETHACIN

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Abstract—Analysis of the effect of a new nonsteroidal anti-inflammatory compound, fenclorac (α,*m*-dichloro-*p*-cyclohexylphenylacetic acid), on prostaglandin biosynthesis *in vitro* has shown the drug to be a potent inhibitor of this enzyme system. Fenclorac was ten times as active as indomethacin in inhibiting prostaglandin synthesis in bovine seminal vesicle microsomes. Inhibition by fenclorac was not appreciably dependent upon preincubation of drug with enzyme for maximal activity, and was reversible by dilution. The inhibitory activity of indomethacin was time-dependent and not readily reversible. Kinetic analysis indicated that fenclorac is a competitive inhibitor of prostaglandin synthetase, irrespective of preincubation conditions, whereas indomethacin inhibits noncompetitively if preincubated with enzyme, and competitively in the absence of any preincubation. Both drugs protected or stabilized prostaglandin synthetase activity under extended preincubation conditions.

Inhibition of the enzyme systems collectively termed prostaglandin synthetase has gained wide acceptance as the postulated primary mechanism of action of nonsteroidal anti-inflammatory (NSAI) drugs. The inflammatory effects of injected prostaglandins, which include edema [1, 2] erythema [3, 4], pain [5] and fever [6, 7], have provided support for the role of prostaglandins, particularly the E and F series, as mediators of inflammation. Vane [8] first demonstrated that two widely used NSAI drugs, aspirin and indomethacin, inhibited production of prostaglandins by inhibiting the enzyme(s) responsible for their synthesis. Since then, many new anti-inflammatory drugs have been shown to be inhibitors of prostaglandin synthetase both *in vivo* and *in vitro*, in man and in a variety of animal species [9].

Prostaglandins are probably not stored, but are synthetized *de novo* and released. Therefore, inhibition of prostaglandin synthetase should result in a rapid decline of existing levels of prostaglandin and a subsequent decrease in inflammation. Flower and coworkers [10] have reported that clinically effective NSAI agents attain blood levels that are more than sufficient for the *in vivo* inhibition of prostaglandin synthetase.

Fenclorac (α,*m*-dichloro-*p*-cyclohexylphenylacetic acid) is a potent NSAI agent possessing significant antipyretic properties. The anti-inflammatory activity of fenclorac has been demonstrated in carrageenan paw edema (CPE) and adjuvant-induced arthritis. The relative potency of fenclorac in CPE was found to be 13 times that of aspirin, 3 times ibuprofen (Motrin®, Upjohn) and 0.3 times indomethacin (Indocin®, Merck, Sharp and Dohme. Fenclorac was 77 times more effective than aspirin and twice as effective as indomethacin in reducing fever in hyperthermic rats. The purpose of this research was to determine the effect of fenclorac on prostaglandin biosynthesis *in vitro*, in order to establish its potency relative to other NSAI drugs, and to attempt to partially characterize the mechanism of *in vitro* inhibition of prostaglandin synthetase.

MATERIALS AND METHODS

Chemicals. The following compounds were supplied by their respective manufacturers: indomethacin (Merck, Sharp and Dohme), ibuprofen (Upjohn), naproxen (Syntex), fenoprofen (Eli Lilly), aspirin, U.S.P. (Monsanto). All drugs were dissolved in propylene glycol (Baker Chemical). Arachidonic acid (5,8,11,14-eicosatetraenoic acid, 99% pure) was purchased from Sigma Chemical Co., 1-epinephrine from Mann Research Labs, and reduced glutathione from Calbiochem. All other chemicals used throughout this study were of reagent grade quality.

Preparation of enzyme. A prostaglandin synthetase enzyme derived from bovine seminal vesicle microsomes was utilized for the *in vitro* synthesis of PGE_2 and $PGF_{2\alpha}$ according to the method of Takeguchi *et al.* [11], and Flower *et al.* [12]. Frozen bovine seminal vesicles (Pel-Freeze Biologicals) were allowed to thaw, and then cleaned of excess fat and connective tissue. Batches of 200–300 g of tissue were divided into 50 g portions, minced by hand and homogenized in 3 vol of 0.1 M Tris–HCl buffer (pH 8.2) in a Sorvall Omnimixer for 2 min. The homogenates were pooled and centrifuged at 18,000 *g* for 10 min at 4°. The resultant supernatant fraction was filtered through 2 layers of cheese-cloth. The filtrate was then centrifuged in a Type 50.2 ti rotor at 105,000 *g* for 1 hr at 4° in a Beckman LS-50 ultracentrifuge. The resultant pellets were removed from the centrifuge tubes with buffer, pooled and hand homogenized in a Potter–Elvehjem glass homogenizer equipped with a teflon pestle. The homogenized microsomal pellets were then lyophilized in 3 ml aliquots

and stored at −70 until required for assay. The protein content, measured by the method of Lowry *et al.* [13], ranged between 510–680 μg/mg of lyophilized powder.

Assay system. The lyophilized powder was reconstituted in 0.1 M Tris buffer (pH 8.0) just before use. Each standard incubation system contained a final concentration of: 50 mM Tris–HCl (pH 8.0), 5 mM glutathione and 5 mM 1-epinephrine, 330 μg microsomal protein and approximately 0.3 μCi of (6,8,9,11,12,14,15)-[^{3}H]arachidonic acid in a final vol. of 0.5 ml. The tritiated arachidonic acid (in hexane) was first evaporated to dryness under nitrogen and then reconstituted with a 200 μM solution of unlabeled arachidonic acid in 0.01 M Tris–HCl buffer (pH 8.0) to a concentration of 6 μCi/ml just prior to use.

Stock solutions of NSAI compounds were prepared in propylene glycol, and were then serially diluted with propylene glycol such that 25 μl of working solution would produce the desired final concentration of drug when diluted into the 500 μl final vol. of the incubate. Routinely, the drug to be tested was introduced into the reaction medium and preincubated with the enzyme in the absence of substrate for 2 min at 37°. The substrate was then added and the reaction allowed to proceed for 4–5 min before being terminated. However, the order in which the components of the enzyme system were added (and hence, the initiation of the reaction) was varied depending upon the type of study being performed. The reactions were performed at 37° in a Dubnoff incubator-shaker. Control incubations contained equal vol. of vehicle. This vol. of propylene glycol usually produced a slight increase in prostaglandin synthesis (10–15%), an effect possibly due to the partial solubilization of microsomal (particulate) bound enzyme.

The reaction was terminated with 0.25 ml 1N HCl. The prostaglandin products and unconverted arachidonic acid were extracted from the incubate with 1.5 ml ethyl acetate. Approximately 95 per cent of the [^{3}H]arachidonic acid and 90 per cent of [^{3}H]PGE_2 and [^{3}H]$PGF_{2\alpha}$ were extracted by mixing the reaction tubes for 20 sec on a "Vortex" type mixer. Following centrifugation, exactly 1.0 ml of ethyl acetate extract was transferred to tubes containing PGE_2 and $PGF_{2\alpha}$ (Analabs, Inc.) and evaporated to dryness under nitrogen. The unlabeled PGE_2 and $PGF_{2\alpha}$ were added to the extract to facilitate visualization of the prostaglandins on the chromatography plates. The residue was redissolved in 50 μl of ethyl alcohol and 10 μl of each sample was spotted on silica gel GF thin layer chromatography plate (Analtec, Inc.) and developed in ethyl acetate–water–iso-octane–acetic acid (11:10:5:2, upper phase following equilibration for 24 hr). Spots were visualized by exposure to iodine vapor and areas with R_f values corresponding to PGE_2 (R_f 0.26) or $PGF_{2\alpha}$ (R_f 0.18) markers were scraped directly into liquid scintillation vials; 0.7 ml of water was added followed by the addition of 15 ml PCS® scintillation fluid (Amersham/Searle). The samples were mixed and radioactivity counted in a Beckman LS-350 liquid scintillation counter, at a counting efficiency of 36 per cent. Counts were corrected for background (30 cpm) and quench by the external standard channels-ratio method. Results are expressed as dis/min.

RESULTS AND DISCUSSION

The effect of incubation time, pH and substrate concentration on prostaglandin synthetase was determined prior to evaluation of drug activity. The conversion of arachidonic acid to PGE_2 and $PGF_{2\alpha}$ was essentially complete in 6–8 min (Fig. 1a). The pH optimum was 7.7–8.1 (Fig. 1b), which is in agreement with results obtained by Flower *et al.* [12]. Measurement of initial reaction velocity at increasing substrate concentrations indicated that the pattern of PGE_2 and $PGF_{2\alpha}$ synthesis differed, and that substrate concentrations above 100 μm are inhibitory to PGE_2 production (Fig. 2a). This apparent inhibition of prostaglandin synthetase by arachidonic acid substrate has been previously reported [12], but at higher substrate levels. The linearity of the reaction at relatively low substrate concentrations (5–20 μm) is shown in Fig. 2b. As a result of these preliminary observations, our standard reactions were incubated for 4 min at pH

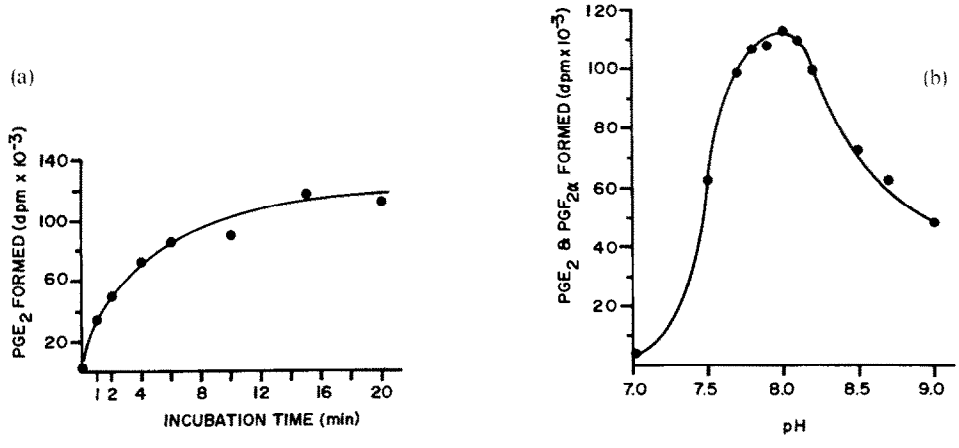

Fig. 1. (a) Time course of the conversion of arachidonic acid into PGE_2 and (b) pH optima curve for the production of total (PGE_2 and $PGF_{2\alpha}$) prostaglandins. These studies conducted at an initial substrate concentration 20 μM.

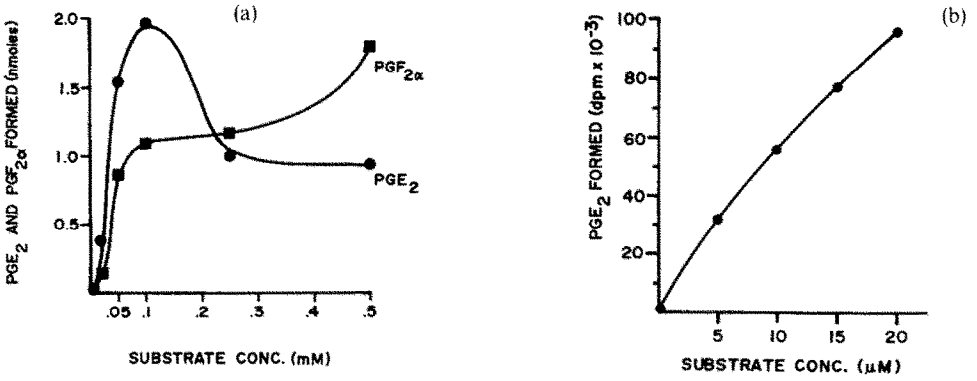

Fig. 2. (a) Effect of arachidonate concentration on the synthesis of PGE_2 and $PGF_{2\alpha}$ by bovine seminal vesicle microsomes, and (b) formation of PGE_2 at low substrate concentration. Incubations were carried out for 4 min.

8.0 at a substrate concentration of 20 μm. This concentration more closely approximates levels of substrate believed to exist *in vivo* [14]. The substrate concentration of the enzyme reaction is relevant in determining the relative potency of NSAI drugs, since several workers have emphasized that the absolute ID_{50} value obtained for many NSAI drugs is dependent upon initial substrate concentrations [9, 14–16].

The concentration of drug required to inhibit enzyme activity by 50 per cent (ID_{50}), was determined graphically. Drugs were preincubated with enzyme for 2 min in the absence of substrate. Under these conditions, the ID_{50} of fenclorac was 0.05 μm and 0.65 μm for indomethacin (Fig. 3). The observed ID_{50} for indomethacin is in agreement with previously reported values obtained from synthetase systems at comparable substrate concentrations [14, 17].

The ID_{50} of other known NSAI drugs, including several recent additions to the clinical compendium, was determined under identical preincubation conditions (Table 1). Fenclorac was the most potent inhibitor of prostaglandin synthetase of the compounds tested. The potency of fenclorac was 10 times indomethacin in inhibiting prostaglandin synthetase *in vitro*. The major plasma metabolite of fenclorac, *m*-chloro-*p*-cyclohexylphenylglycolic acid, possessed slightly greater inhibitory potency than indomethacin. It must be emphasized, however, that the ID_{50} values of inhibitors are totally dependent upon initial substrate concentration, preincubation time, and other incubation conditions [9, 12, 15].The relevance of *in vitro* ID_{50} values to *in vivo* drug activity should be viewed in terms of the absence of any *in vivo* preincubation conditions due to the continued presence of endogenous substrate.

Several reviewers [9, 15, 18] have emphasized the rank order correlation between the ID_{50} values for prostaglandin synthetase inhibition and the ED_{50} values obtained in the carrageenan paw edema assay for NSAI drugs. However, our studies demonstrated that the wide difference in synthetase ID_{50} values between fenclorac and indomethacin was not apparent when these drugs were tested in the CPE assay system, in which fenclorac is approximately one-third as active as indomethacin, but 4–12 times as active as ibuprofen and aspirin, respectively. It is possible that this apparent lack of correlation may be attributed, at least in part, to the differences in rate of metabolism, protein binding, and subcellular distribution of the two compounds. The plasma elimination half-time of fenclorac is 1.5 hr [19] and 4.0 hr for indomethacin [20] in the rat. In addition, subcellular distribution studies using ^{14}C-labeled fenclorac indi-

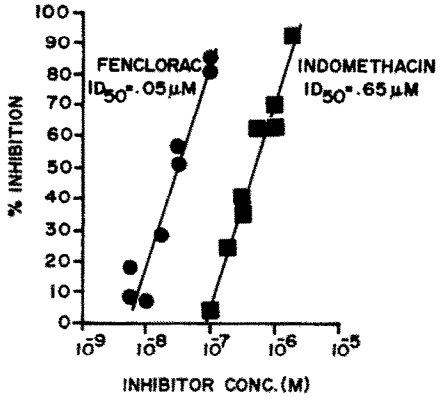

Fig. 3. Inhibition of total prostaglandin synthesis (PGE_2 and $PGF_{2\alpha}$) by fenclorac and indomethacin. Drugs were preincubated with enzyme preparation at 37° for 2 min prior to addition of substrate; (●) fenclorac and (■) indomethacin.

Table 1. Comparative effects of fenclorac and other nonsteroidal anti-inflammatory agents on the *in vitro* inhibition of prostaglandin synthetase and carrageenan-induced paw edema in rats

Compound	Prostaglandin synthetase inhibition ID_{50} (μm)	Paw edema inhibition ED_{50} (mg/kg)
Fenclorac	0.05	7.9
m-chloro-*p*-cyclohexylphenylglycolic acid	0.47	14.7
Indomethacin	0.65	5.0
Naproxen	3.5	3.7
Fenoprofen	5.0	20.3
Ibuprofen	10.0	31
Aspirin	2500	97

cated that greater than 90 per cent of the drug was found in the cytoplasmic fraction with very low concentration in the microsomal fraction of rat hepatocytes (Procaccini, unpublished observations). These findings suggest that *in vivo* binding of a drug to the endoplasmic reticulum, the subcellular location of the target enzyme, may be a critical factor in the correlation of *in vitro* inhibition of prostaglandin synthetase and *in vivo* anti-inflammatory activity.

Certain *in vitro* inhibitors of prostaglandin synthetase require a preincubation period, whereby the drug is allowed to react with the enzyme *in vitro* prior to the addition of substrate. Maximal inhibition by indomethacin and aspirin is attained only if the incubation components are added in this manner. In our studies, several concentrations of either fenclorac or indomethacin were preincubated with enzyme, cofactors and buffer, but in the absence of substrate, for 0–8 min at 37°, prior to the addition of arachidonic acid. The reaction with substrate was allowed to proceed for an additional 4 min before being terminated with hydrochloric acid. The data presented in Fig. 4 show that the maximal inhibiton (35–40 per cent) demonstrated by fenclorac at a concentration of 0.025 μm was relatively independent of preincubation time. However, inhibition by indomethacin (0.5 μm) ranged about 26 per cent with no preincubation to over 65 per cent following a 6 min preincubation, thus demonstrating a clear preincubation time dependency for this drug. These findings were not unexpected, since other workers [19, 21], have shown that 5–10 min is required for full inhibition to occur when indomethacin was added directly to a prostaglandin synthesizing system.

When the time of drug preincubation was extended to 15 min, an apparent elevation in enzyme activity above controls occurred with fenclorac, but not indomethacin (Fig. 5). The relative enzyme activity would indicate that fenclorac actually activated the enzyme preparation during the preincubation period, when substrate was not present. The reason for this observation was clarified by the finding that preincubation of the enzyme preparation at 37°, in the absence of arachidonic acid or drugs, resulted in a significant time-dependent loss of activity (Fig. 5a). The control preparation, which was incubated simultaneously with the fenclorac samples, lost approximately 74 per cent of its initial activity following 15 min preincubation, presumably because of inactivation due to enzyme instability in the absence of substrate (Fig. 5a).

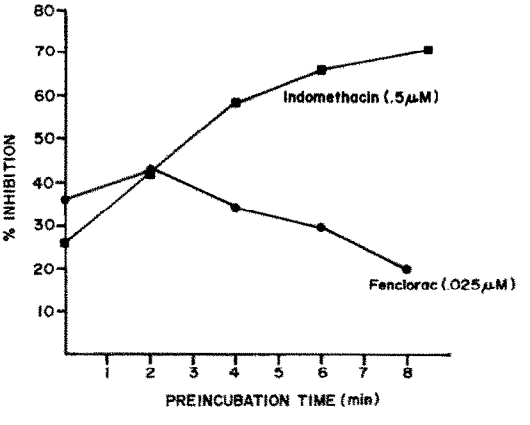

Fig. 4. Effect of preincubation time on the inhibition of PGE_2 production by fenclorac (●) and indomethacin (■). Drugs were preincubated with enzyme for the indicated time period prior to the addition of arachidonate.

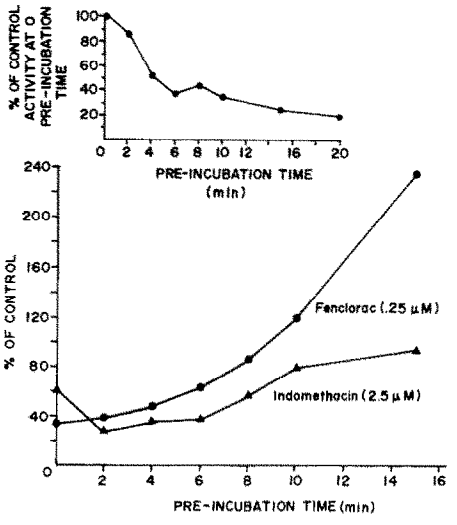

Fig. 5. Effect of extended preincubation on PGE_2 synthesis by fenclorac (●) and indomethacin (▲). Each drug was preincubated with enzyme in the absence of substrate for the indicated time period. Incubations were carried out for 4 min following addition of substrate. (a) Inset plot demonstrates the loss of activity with time when the enzyme is preincubated without drug or arachidonic acid.

These data indicate that the prostaglandin synthetase preparation utilized in these studies is unstable when incubated at 37° only in the absence of substrate. A similar loss of enzyme activity during preincubation of a sheep vesicular gland synthetase preparation at 30° in the absence of arachidonate was noted in a report by Smith and Lands [21]. Only 5 per cent of the initial enzyme activity remained after 5 min preincubation of their enzyme preparation in the absence of substrate. On the other hand, van den Berg *et al.* [22] reported that 5 min preincubation of enzyme at 37° in the absence of substrate did not affect enzyme activity. Differences in both enzyme preparation and incubation conditions may account for these discrepancies in observations.

Our results suggest that the overall effect of incubation of enzyme with fenclorac in the absence of substrate may be biphasic. The initial effect of enzyme inhibition is predominate during the first 6 min of incubation (Fig. 5). However, upon extended incubation periods, the inhibition is apparently reversed, i.e., following 8–10 min preincubation (Fig. 5), fenclorac did not inhibit enzyme activity relative to preincubated controls (Fig. 5a). Further extension of the preincubation time resulted in prostaglandin synthetase activity which was higher in samples containing fenclorac than was found in preincubated control samples. It should be noted that the apparent elevation (240 per

cent of control) in activity following a 15 min preincubation with fenclorac in the absence of substrate results simply from recovery of approximately 62% of activity in these samples (Fig. 5) vs only 26 per cent of activity recovered from the 15 min preincubated control samples (Fig. 5a). This effect was not as apparent in enzyme samples preincubated with indomethacin under the same conditions. These results suggest a drug-induced stabilization and/or protection of the enzyme under conditions where inactivation would normally occur.

The apparent reversibility of the inhibition of prostaglandin synthetase was determined using a dilution-centrifugation procedure similar to that described by van den Berg [22]. Two series of incubations, were prepared. The first series of incubations (series I) contained 4–5 times ID_{50} concentrations of either fenclorac or indomethacin preincubated with enzyme at 37° for 2–10 min prior to the addition of substrate. At the specified times, substrate was added and the reaction was carried out for 5 additional min before being terminated with acid. The data from this study was used as a test to verify that these drug concentrations were capable of completely inhibiting total enzyme activity under standard preincubation conditions. In a parallel series of samples (series II), enzyme, buffer and either propylene glycol (drug vehicle), fenclorac, or indomethacin, at the same concentrations used in the first series, were preincubated for 2–10 min in the absence of substrate. The reaction was then terminated nondestructively by adding 8 vol of cold 0.1 M Tris buffer to the incubation. The diluted incubations were centrifuged immediately at 105,000 *g* for 1 hr in a Beckman L5-50 ultracentrifuge (SW 50.2 ti rotor) in order to pellet and reisolate the enzyme. The supernatant wash, which contained excess drug, was decanted and the tubes were rinsed 2× with buffer. The enzyme was resuspended with buffer and cofactors, substrate was added to initiate the reaction without any further addition of drug, and the remaining enzyme activity analyzed as previously described. The results obtained from both series are presented in Fig. 6.

In series I (Fig. 6, closed symbols), preincubation of enzyme with excess ID_{50} concentrations of either fenclorac or indomethacin for 2–10 min resulted in complete inhibition of prostaglandin synthetase activity. However, in series II (Fig. 6, open symbols), preincubation of enzyme with fenclorac for 2 min, followed by removal of drug by the dilution-centrifugation procedure and analysis of remaining enzyme activity, resulted in recovery of approximately twice the control enzyme activity (Fig. 6, open symbols). It should be noted that 50–75 per cent of the initial enzyme activity was lost between the first and second control incubations, due both to normal denaturation of the enzyme during the 2 hr required for washing and reassay procedures, and to the previously observed loss of activity during the incubation of enzyme in the absence of substrate (Fig. 5a). The level of recoverable enzyme activity in series II was found to be dependent upon the duration of initial preincubation, i.e., after preincubation of enzyme with fenclorac for 10 min, synthetase activity measured after removal of the drug by dilution-centrifugation was approximately 90 per cent of control levels. In contrast, following 10 min preincubation with indomethacin, significant enzyme inhibition remained even after the drug was removed from the system. These results are in general agreement with those reported for indomethacin [22] under similar preincubation conditions. However, approximately 140 per cent of control activity was recovered from enzyme preincubated with indomethacin for 2 min, followed by removal of the drug by the dilution-centrifugation procedure. This action has not been previously reported with indomethacin.

Although the protective effect of both drugs observed at 2 min preincubation is lost or reversed on extended preincubation (Fig. 6), the mechanisms by which each drug affects these changes are probably different. In the case of fenclorac, the reversible inhibition may be due to displacement of drug from the enzyme, resulting in the loss of enzyme inhibition, whereas, the inhibitory effects of indomethacin become more pronounced as the initial preincubation period is increased. The results of this study indicate that the inhibition of prostaglandin synthetase by fenclorac can be reversed by simply washing the microsomal pellet to remove the drug, whereas, the inhibition produced by indomethacin is not completely reversible under identical conditions.

In order to determine the type of inhibition produced by fenclorac, enzyme activity was measured at various concentrations of substrate and inhibitor. Initially, a preincubation period was not utilized, since results obtained from our preincubation studies indicated that this procedure did not appreciably affect the degree of inhibition with this drug. A Lineweaver–Burk plot of the data is presented in Fig. 7a. The apparent K_m of the enzyme observed in our studies is 5.7×10^{-5} M as compared to K_m values of 1.65×10^{-5} M, 7.0×10^{-5} M, and 8.2×10^{-5} M reported by Ho and Esterman [14], Takeguchi *et al.* [11], and van den Berg *et al.* [22], respectively. Under the conditions employed in this study (no preincuba-

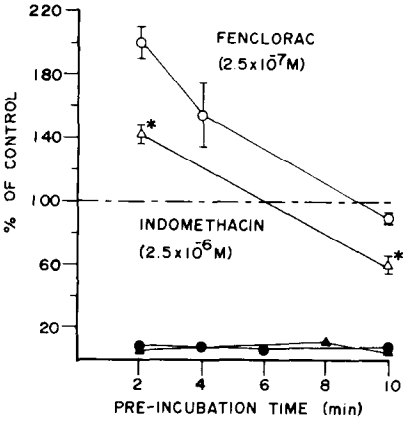

Fig. 6. The reversibility of prostaglandin synthetase inhibition by fenclorac and indomethacin: Closed symbols represent enzyme activity obtained from unwashed drug-treated microsomes (series I), whereas open symbols represent enzyme activity recovered from washed microsomes (series II). Each point represents the mean ± S.E. of combined data from three experiments. (●, ○—fenclorac; ▲, △—indomethacin.) * Significantly different ($P < 0.01$) from control and fenclorac-treated samples.

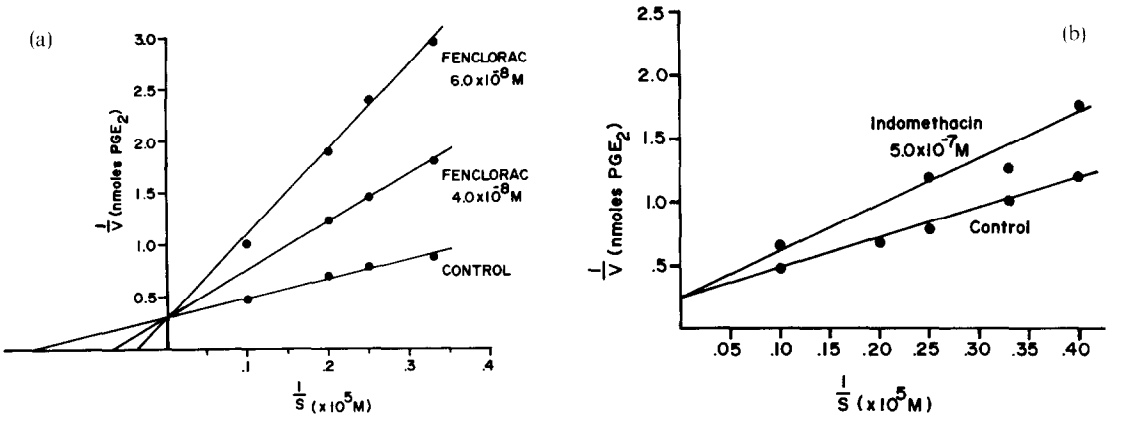

Fig. 7. Lineweaver–Burk plots of the inhibition of PGE_2 synthetase by (a) fenclorac and (b) indomethacin. Incubations were initiated by the addition of enzyme without drug preincubation.

tion), fenclorac exhibited apparent competitive inhibition of the enzyme system. Indomethacin demonstrated a clearly competitive effect when analyzed under identical conditions (Fig. 7b). Previous investigations [14, 16, 21] have reported noncompetitive inhibition with indomethacin.

The work of Ho and Esterman [14] and Lands *et al.* [23] have demonstrated that NSAI drugs inhibit prostaglandin synthetase in a biphasic manner. The terminology used by recent reviewers is "competitive-irreversible inhibition". This concept is based upon the proposed mechanism wherein the inhibitor binds to the enzyme at a site in close enough proximity to the catalytic site to decrease the activity of prostaglandin synthetase in a time-dependent manner. The inhibitor binds only to free enzyme not associated with substrate. However, once the enzyme is attacked by the inhibitor, the enzyme is irreversibly blocked. Ultimately, with time, enough enzyme will be irreversibly inhibited to stop further conversion of substrate. From this model, it is assumed that the extent of inhibition will be dependent upon initial substrate concentration as proposed by Flower [9], and that the extent of irreversible inhibition will increase with time. If this mechanism is correct, then an initial competitive inhibition of prostaglandin synthetase by indomethacin would not be unexpected. Several reports suggest that the inhibition of prostaglandin synthesis by NSAI drugs, including aspirin and indomethacin, is time-dependent and is enhanced by preincubation [22, 24]. The present studies indicated that indomethacin-mediated inhibition was virtually complete (maximal) at 4–6 min. Analysis of the inhibition produced by fenclorac and indomethacin with a 6 min preincubation period indicated that increasing the preincubation time from 0 to 6 min did not alter the competitive nature of fenclorac-mediated inhibition (Fig. 8a). However, as reported by other workers, the inhibition by indomethacin following a 6 min preincubation was apparently noncompetitive (Fig. 8b). Therefore, the type of inhibition elicited by indomethacin in this system was strictly dependent upon the length of preincubation. These results support the concept that the initial inhibition produced by indomethacin is competitive, whereas in the latter phases of the reaction, inhibition is noncompetitive (time-dependent, irreversible). The nature of fenclorac inhibition, however, was not altered by preincubation and is apparently competitive throughout the reaction. The fact that fenclorac-mediated inhibition of prostaglandin synthetase was competitive supports the contention that the apparent protection of the enzyme by fenclorac during either prolonged preincubation without substrate (preincubation study) or during the

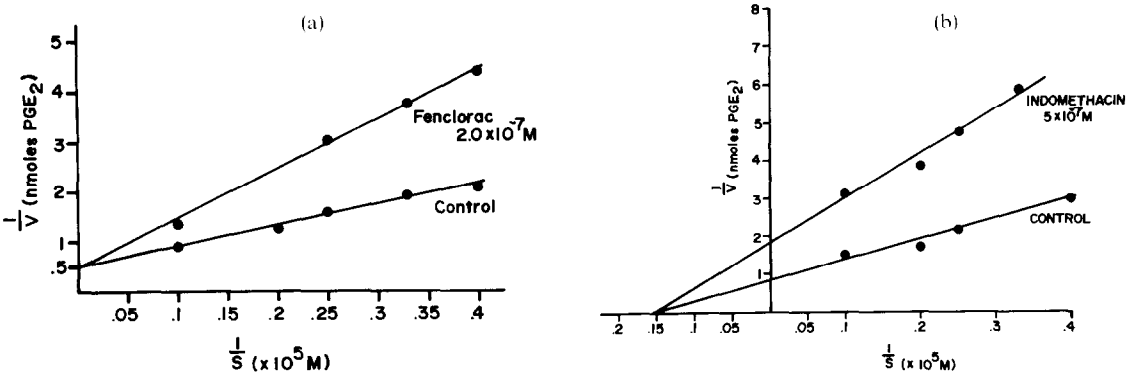

Fig. 8. Lineweaver–Burk plots of the inhibition of PGE_2 synthesis by (a) fenclorac and (b) indomethacin. Drugs were preincubated with enzyme for 6 min prior to substrate addition.

dilution-centrifugation procedure (reversibility study) may be due to the binding of the drug directly to the catalytic site of the enzyme, which is normally occupied by arachidonic acid. The partial stabilization/protection of the enzyme by indomethacin following a 2 min preincubation period (Fig. 6) may be explained similarly, since the early phase of indomethacin inhibition is also competitive in nature. Initially, indomethacin would presumably react with the enzyme in a similar, but not identical manner as fenclorac, leading to stabilization followed by irreversible inhibition as the preincubation time is increased (Fig. 6). It is to be emphasized that this action is observed only when the drug is removed from the previously inhibited enzyme. The fact that the enzyme preparation loses significant activity when preincubated at 37° without substrate, but is stable in the presence of fenclorac (Fig. 5) or arachidonic acid again suggests that fenclorac partially protects the enzyme by binding to the same site on the enzyme as does arachidonic acid. Several enzymes, including mitochondrial DNA polymerase [25], and microsomal glucose-6-phosphatase [26] are inactivated when incubated at 37° in the absence of substrate, but are stabilized in the presence of substrates or inhibitors. The stabilization and/or protection of prostaglandin synthetase activity was also reported with two members of a series of 2-aryl-1,3-indanediones, both of which were active *in vitro* inhibitors of prostaglandin synthetase [22].

In summary, these studies indicate that fenclorac blocks prostaglandin synthetase, but that the mechanism(s) by which this drug inhibits the enzyme differs from indomethacin. This conclusion is based on the findings that fenclorac-mediated inhibition does not require preincubation, is almost completely reversible, and demonstrates competitive-type kinetics throughout extended preincubation periods. In contrast, indomethacin-mediated inhibition requires a preincubation time for maximal activity, is reversible only during the initial preincubation stages, and demonstrates noncompetitive kinetics during extended (>6 min) preincubation periods. The time-dependent (reversible) stabilization of prostaglandin synthetase mediated by both drugs during the initial phase of the drug-enzyme interaction, suggests that these drugs may bind at, or in close proximity to, the active site of the enzyme.

Fenclorac is a potent competitive inhibitor of prostaglandin synthetase *in vitro* and may be included in the list of drugs which possess anti-inflammatory activity *in vivo*, which is partially mediated by their action on this enzyme system. Furthermore, fenclorac, indomethacin, and possibly other nonsteroidal anti-inflammatory drugs possess the interesting property of stabilizing this enzyme system under certain *in vitro* conditions. The applicability of this latter action to *in vivo* drug activity has not been defined.

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