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THE INFLUENCE OF SATURATED FATTY ACIDS ON PROSTAGLANDIN SYNTHETASE ACTIVITY

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Abstract—Saturated fatty acids (C_{10}, C_{12}, C_{14}, C_{16} and C_{18}) as well as lauryl sulphate inhibit the microsomal prostaglandin synthetase of bovine seminal vesicles (BSVM). The most potent inhibitors are lauryl sulphate, lauric and myristic acids (IC_{50} = 250 μM). The last two acids are strong ligands to hydrophobic sites of albumin. Indomethacin is also strongly bound to the hydrophobic sites of albumin; however, indomethacin inhibits the generation of prostaglandins at a concentration approximately 2500 times lower than the most active fatty acid inhibitor. The inhibitory action of fatty acids and indomethacin on prostaglandin synthetase activity has been measured by estimation of either PGE_2 or malondialdehyde, which are generated from arachidonic acid by BSVM. The former procedure is more reliable and reproducible than the latter.

The inhibitory action of unsaturated fatty acids on prostaglandin (PG) synthetase has been thoroughly investigated [1, 2] and reviewed [3], while the influence of saturated fatty acids on the activity of this microsomal enzyme is hardly mentioned in the literature [4]. Here we report on the influence of seven saturated fatty acids (C_6–C_{18}) on the generation of prostaglandin E_2 (PGE_2) and malondialdehyde from arachidonic acid by bovine seminal vesicle microsomes (BSVM). We also describe the ability of these acids to displace 8-anilino-1-naphthalene sulphonate (ANS) from hydrophobic sites of bovine serum albumin. Generation of malondialdehyde from arachidonic acid by the microsomal enzyme has been described by Samuelsson [5] and proposed [6] as the index of prostaglandin synthetase activity. Binding of PG synthetase inhibitors to hydrophobic sites of albumin has been reported previously [7, 8].

MATERIALS AND METHODS

The following substances were used: Bovine serum albumin (Cohn fraction V), produced by Polish Serum and Vaccines Works (dialized and lyophylized 95–99% albumin); 8-anilino-1-naphthalene sulphonic acid ammonium salt (ANS), kindly supplied by Sigma Chemical Co.; Arachidonic acid, Sigma; Glutathione, produced by Polish Serum and Vaccines Works; Hydroquinone, Malinckrodt; Adrenaline, British Drug Houses; Malondialdehyde tetramethylacetal, K and K Laboratories Inc., Plainview; Sodium lauryl sulphate, BDH; Indomethacin, Polfa; Hexanoic acid (caproic), Riedel de Haen A.G. Seelze; Octanoic acid (caprylic), Koch and Light Laboratories; Decanoic acid (capric), Reachim; Dodecanoic acid (lauric), Schuchardt; Tetradecanoic acid (myristic), Koch and Light Labs; Hexadecanoic acid (palmitic), Schuchardt; Octadecanoic acid (stearic), Schuchardt.

Solutions of sodium salts of fatty acids were made up with a stechiometric volume of 1 N NaOH, heated, and diluted with distilled water.

Prostaglandin synthetase activity. Arachidonic acid was used as the substrate, glutathione (160 μM) and hydroquinone (45 μM) as cofactors, and BSVM (1 mg protein/ml) as the source of the enzyme. The final volume of samples was 2 ml in 0·067 M phosphate buffer pH 8. In kinetic experiments (calculation of K_m and K_i values) the substrate concentration was 10–1000 μM, while in other experiments arachidonic acid was used at a concentration of 33 μM. The sequence of composition of the reaction mixture was: arachidonic acid, activators, an inhibitor and the enzymic preparation. Samples were incubated for 5 min and then boiled. The enzymic activity was measured by bioassay of the end product of the reaction i.e. PGE_2 [6]. We found previously [7, 9], using two systems of thin layer chromatography, that in abovementioned experimental conditions 80–90% of biological activity in the incubation mixture is confined to PGE_2.

Solutions of sodium salts of saturated fatty acids were found not to interfere with PG bioassay. The potency of myristic acid to inhibit PG synthetase by 50% (IC_{50}) was calculated from the regression equation, where x is logarithm of myristic acid concentration (10–1000 μM), and y is per cent of enzyme inhibition. IC_{50} values for other fatty acids were estimated graphically. Reference substances (lauryl sulphate and indomethacin) were tested at four concentrations and regression lines were calculated.

Malondialdehyde formation. The formation of malondialdehyde from arachidonic acid (150–1000 μM) by lyophylized BSVM was determined by the method of Flower *et al.* [6]. The inhibitors (indomethacin and myristic acid) were not preincubated with BSVM. The sequence of preparation of the incubation mixture was the same as for the estimation of PG synthetase activity. The amount of malondialdehyde formed was read from the standard curve drawn for malondialdehyde tetramethylacetal.

Binding of 8-anilino-1-naphthalene sulphonate (ANS) to bovine serum albumin. Fluorescence of the complex of ANS with albumin was measured at 470 nm (exci-

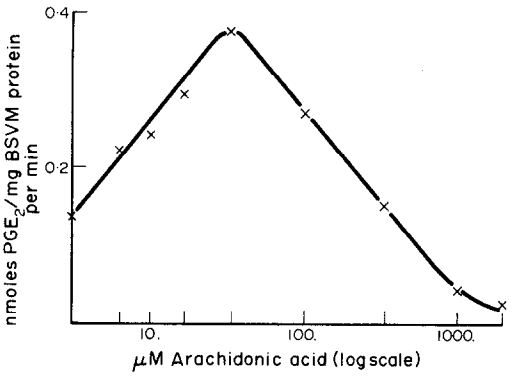

Fig. 1. Influence of the substrate concentration on velocity of prostaglandin E_2 formation by bovine seminal vesicle microsomes (BSVM). The composition of the reaction mixture was as follows, 10–2000 μM arachidonic acid, 45 μM hydroquinone 160 μM glutathione, 1 mg BSVM protein/ml in 0·067 M phosphate buffer pH 8·0. The final volume of the incubation mixture was 2 ml.

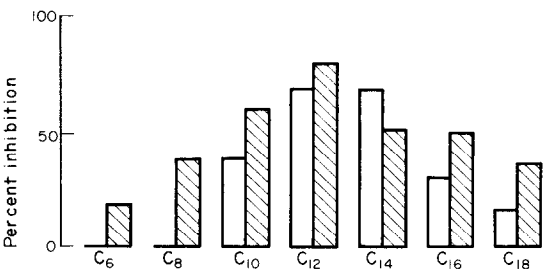

Fig. 2. Inhibition of prostaglandin synthetase activity (white columns) and quenching of the fluorescence of the complex of 8-anilino-1-naphthalene sulphonate (ANS) with albumin (striped columns) by saturated fatty acids at a concentration of 10^{-3} M. Inhibition of prostaglandin synthetase activity was investigated in the same conditions as those referred in Fig. 1, except for the concentration of arachidonic acid which was 33 μM. Fluorescence was measured in a solution containing 10 μM bovine serum albumin and 40 μM ANS (excitation wavelength 380 nm, fluorescence 470 nm).

tation wavelength 380 nm). Quenching of fluorescence by the saturated fatty acids and by the reference substances was estimated by the method reported previously [8].

RESULTS

A strong inhibition of enzymic PGE_2 generation by an excess of the substrate was observed (Fig. 1). The K_m value was $7{\cdot}6 \times 10^{-6}$ M (Fig. 3).

The potencies of saturated fatty acids to inhibit PG synthetase activity and to quench the fluoresence of the ANS–albumin complex (Fig. 2) increased (for C_6–C_{12} acids) and decreased (for C_{14}–C_{18} acids) in parallel. The IC_{50} values for inhibition of PG synthetase and for ANS displacement from albumin by myristic acid were compared with the corresponding IC_{50} values for reference substances, i.e. indomethacin and lauryl sulphate (Table 1).

Indomethacin is a much stronger inhibitor of PG synthetase than myristic acid and lauryl sulphate (Table 2) whereas the binding to albumin hydrophobic sites is of the same order of magnitude for all three compounds. The kinetic data for inhibition of PG synthetase (Fig. 3) revealed that indomethacin is a competitive inhibitor of PG synthetase ($K_i = 3{\cdot}6 \times 10^{-8}$ M) providing that the drug has not been preincubated with the enzyme. When indomethacin was preincubated with BSVM for 5 min before the substrate was added, then the inhibition of enzymic activity also occurred, but independently of the concentration of arachidonic acid used. Inhibition of the enzymic activity by myristic acid seemed to be of mixed type (Fig. 3).

The generation of malondialdehyde by BSVM was estimated at concentrations of arachidonic acid from 150 uM to 1000 μM; however, a linear increase in velocity of the reaction was observed in a range of concentrations from 300 to 600 μM of the substrate (Fig. 4). The approximate K_m value was 4×10^{-4} M and

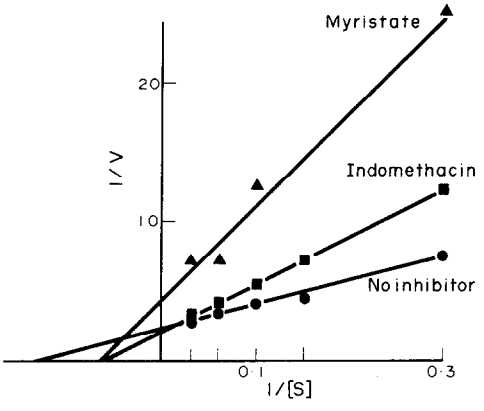

Fig. 3. Lineweaver and Burk's double-reciprocal plot for inhibition of prostaglandin synthetase activity by myristate and indomethacin. The lines were drawn by the method of least squares. The experimental conditions were the same as in Fig. 1. K_m was $7{\cdot}6 \times 10^{-6}$ M in the absence of an inhibitor and 17×10^{-6} M in the presence of indomethacin at a concentration of 8×10^{-8} M. V_{max} was 0·448 nmoles PGE_2/mg of BSVM protein/min in the absence of inhibitor and 0·503 in the presence of indomethacin. In the presence of myristate at a concentration of 10^{-3} M K_m was $15{\cdot}5 \times 10^{-6}$ M and V_{max} was 0·226 nmoles PGE_2/mg BSVM protein per min. Each point represents the mean of 3–4 experiments.

Table 1

Compounds	IC_{50} for ANS displacement (μM)	IC_{50} for PG synthetase inhibition (μM)
Myristic acid	260	265
Indomethacin	140	0·07
Lauryl sulphate	100	250

Table 2. Inhibition of prostaglandin E_2 generation from arachidonic acid by bovine seminal vesicle microsomes (BSVM)

Inhibitor	IC_{50} (μM)	n (number of experiments)	b (slope)	a (intercept)	r (regression coefficient)	S.D.
Indomethacin	0·07	23	49·6	−21·19	0·8643	9·21
Lauryl sulphate	250	13	39·7	−143·11	0·9050	9·53
Myristate	265	30	30·5	−95·7	0·7327	13·3

The composition of the reaction mixture was the same as in Fig. 1 but the concentration of arachidonic acid was 33 μM. The potency of indomethacin, lauryl sulphate and myristate to inhibit prostaglandin synthetase activity by 50% (IC_{50}) was calculated from the regression equation $y = a + bx$ where y is per cent of inhibition of enzyme activity and x is log of concentration of an inhibitor (ng/ml).

V_{max} about 0·69 nmoles of malondialdehyde/mg BSVM protein per min. Myristic acid and indomethacin seem to be noncompetitive inhibitors of malondialdehyde formation. The approximate K_i values for myristic acid and indomethacin were 10^{-2} M and 7×10^{-5} M respectively. It was not possible to calculate K_i by the method of Lineweaver and Burk because the curves did not cross the $1/v$ axis. Therefore, we approximated these values as follows. V_{max} was assumed as the velocity of the reaction at a concentration of arachidonic acid of 700 μM, since further increase of the substrate concentration did not increase the velocity. In the absence of inhibitors K_m was found on [S] axis for $1/2\,V_{max}$. Both inhibitors changed only V_{max} but not K_m and therefore K_i values were calculated from the formula:

$$K_i = \frac{[I]}{\frac{V_{max}}{V_i} - 1}$$

where [I] is inhibitor concentration, V_{max} is the velocity of the reaction in the absence of inhibitor and V_i is the velocity of the raction in the presence of inhibitor.

DISCUSSION

The inhibition of the enzymic formation of PGE_2 in bovine seminal vesicle microsomes (BSVM) by an excess of arachidonic acid has been reported by Flower *et al.* [6]. The K_m value calculated from their graph is about 10^{-4} M. Ho and Esterman [10] have reported K_m equal to $1{\cdot}65 \times 10^{-5}$ M. Our K_m value ($7{\cdot}6 \times 10^{-6}$ M) is nearer the latter result. The divergence in K_m values obtained in different laboratories may result from a different composition of reaction mixtures. Flower *et al.* [6] used very high concentrations of glutathione (5000 μM) and adrenaline (5000 μM); Ho and Esterman [10] used 3000 μM of glutathione and no adrenaline; while in this paper 160 μM of glutathione and 45 μM of hydroquinone were used.

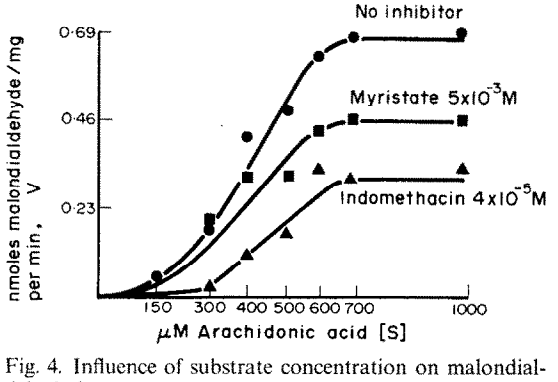

Fig. 4. Influence of substrate concentration on malondialdehyde formation in the absence or in the presence of indomethacin and myristate. The composition of the reaction mixture was as follows: 5000 μM adrenaline, 5000 μM glutathione, 300–1000 μM arachidonic acid and 1 mg BSVM protein/ml in 0·1 M Tris buffer pH 8·2. The final volume of the incubation mixture was 2 ml.

We have confirmed the results of Wallach and Daniels [4] concerning the inhibitory action of capric acid on PG synthetase although its inhibitory potency is somewhat weaker than that of lauric acid, which in turn is reported by Wallach and Daniels [4] to be inactive as the inhibitor of PG synthetase. Wallach and Daniels [4] used a different enzyme preparation (sheep seminal vesicle microsomes) and a low substrate concentration (1 μM). Possibly this may explain the differences between their results and ours.

In the investigated series of saturated fatty acids lauric and myristic acids are among the most potent ligands to albumin and at the same time both acids are the most potent inhibitors of PG generation. An increase (above C_{14}) or a decrease (below C_{12}) in the length of the carbon chain of fatty acids impairs their interaction with the enzymic and non-enzymic proteins. This observation favours our previous supposition [7] that in homogenous series of the PG synthetase inhibitors an increase in their potency to bind to hydrophobic sites of albumin is usually paralleled by increase of their inhibitory action on the enzymic activity.

Partition coefficients (heptane/water) of saturated fatty acids increase with their chain length [11]. It has been reported that binding to proteins in a homologous series of compounds increases when the apolar part of their molecule is enlarged [12]. Recently however, Cunningham has found [13] that lauric acid is more potent than palmitic acid in displacing tryptophan from albumin. It may be that the length of a 12–14 C chain in the saturated fatty acid molecule is optimal for binding to the hydrophobic sites of enzymic and non-enzymic proteins [9], but it also may be that sodium salts of long-chain fatty acids (>14 C) form micelles [14] in an aqueous medium and so their interaction with protein is impaired.

Table 3

Estimated reaction-product	K_m	K_i for indomethacin	K_i for myristate
Malondialdehyde	4×10^{-4}M	7×10^{-5}M	$\sim 10^{-2}$M
PGE_2	8×10^{-6}M	$3{\cdot}6 \times 10^{-8}$M	—

Myristic acid is about 2500 times less potent than indomethacin as an inhibitor of PG synthetase. Its anti-enzymic potency is similar to that of lauryl sulphate ($IC_{50} = 250\ \mu$M) and aspirin ($IC_{50} = 164\ \mu$M) [9]. Sodium lauryl sulphate has anti-inflammatory action *in vivo* [15], but myristic acid has not been tested as an antiphlogistic agent.

Flower *et al.* [6] have found that the inhibition of PG synthetase by indomethacin is substrate dependent, and Ku and Wasvary [16] reported that the K_i value for indomethacin is $6{\cdot}5 \times 10^{-6}$ M. Our findings indicate that indomethacin is a competitive inhibitor of PG synthetase, provided that the substrate and the inhibitor are added at the same time into the enzymic preparation. Ho and Esterman [10], however, reported that indomethacin is a noncompetitive inhibitor of PG synthetase. These authors used a low substrate concentration (0·1–1·0 μM) and no cofactors were added. We also found that when indomethacin was preincubated with the enzyme, inhibition of enzymic activity was not substrate-dependent. Possibly the inhibition of PG synthetase activity by indomethacin is similar to "active site-directed irreversible inhibition", which has been described [17] for inhibition of adenosine desaminase by adenine derivatives.

The mechanism of PG synthetase inhibition by myristic acid is not clear, since the inhibition of the enzyme occurs at high concentrations of myristic acid and thus its inhibitory action may be unspecific.

The kinetic parameters calculated for malondialdehyde formation differ from those calculated for PGE_2 formation by BSVM (Table 3).

The difference in K_m values confirms the observation of Flower *et al.* [6] that the kinetics of malondialdehyde formation differs considerably from that of PG formation. Indomethacin has lower K_i value for PGE_2 than for malondialdehyde formation.

We found it difficult to reproduce the intensity of malondialdehyde formation from arachidonic acid by BSVM from one experiment to another. This may depend on some non-enzymic formation of malondialdehyde simultaneously with the enzymic one. Discrete changes in concentrations of metal ions, or oxygen in solvents, may influence this reaction. Therefore we cannot recommend this method as an alternative to the direct measurement of prostaglandins formation.

Normal values for free fatty acids in human serum are between 0·1 and 1·0 mM [19] and about half of that amount constitute saturated fatty acid [20]. Because of the binding to albumin, it is hardly possible that a free saturated fatty acid may reach a concentration in serum, which would be inhibitory for PG synthetase in platelets, leucocytes or any other blood cells, providing that PG synthetase in these cells has a similar susceptibility to saturated fatty acids as the BSVM enzyme. Recently it has been reported that the ratio of PGE_2 precursor to PGE_1 precursor (arachidonic acid to dihomo-gamma-linolenic acid) in blood platelets may be a regulatory factor for the intravascular platelets aggregation and thombosis [18]. One can speculate that a dramatic rise in concentration of saturated fatty acids in serum may influence this regulatory mechanism.

Concluding remarks

(1) Saturated fatty acids (C_{10}–C_{18}) at a concentration of 10^{-3} M inhibit the activity of PG synthetase to different degrees; the most potent inhibitors are lauric and myristic acids. Caproic (C_6) and caprylic (C_8) acids are inactive in this respect.

(2) The order of potency to inhibit PG synthetase by saturated fatty acids is the same as the order of their affinity to hydrophobic sites of albumin.

REFERENCES

1. W. E. M. Lands, P. R. Ic Tellier, L. H. Rome and J. Y. Vanderhoek, in *Advances in Biosciences* (Eds. S. Bergström and S. Bernhard), Vol. 9, p. 15. International Conference of Prostaglandins, Vienna. Pergamon Press–Vieweg, Braunschwieg (1973).
2. D. H. Nugteren, *Biochim. biophys. Acta* **210**, 171 (1970).
3. R. J. Flower, *Pharmac. Rev.* **26**, 33 (1974).
4. D. P. Wallach and E. Daniels, *Biochim. biophys. Acta* **231**, 445 (1971).
5. B. Samuelsson, *Fedn Proc.* **31**, 1442 (1972).
6. R. J. Flower, H. S. Cheung and D. W. Cushman, *Prostaglandins* **4**, 325 (1973).
7. J. Robak, A. Dembińska-Kieć, B. Panczenko and R. Gryglewski, *Proc. 2nd Congress Hungarian Pharmacological Soc., Budapest* 1974 (in press).
8. J. Robak, B. Panczenko and R. Gryglewski, *Biochem. Pharmac.* **24**, 571 (1975).
9. R. J. Gryglewski, in *Prostaglandin Synthetase Inhibitors*, (Eds. H. J. Robinson and J. R. Vane), p. 33. Raven Press (1974).
10. P. P. K. Ho and M. A. Esterman, *Prostaglandins* **6**, 107 (1974).
11. De Witt S. Goodman, *J. Am. chem. Soc.* **80**, 3892 (1958).
12. B. B. Brodie and A. M. Hogben, *J. Pharm. Pharmac.* **9**, 345 (1957).
13. V. J. Cunningham, *Abstracts from 9th FEBS Meeting, Budapest*, p. 368. (1974).
14. P. H. Elworthy, A. T. Florence and C. B. Macfarlane, in *Solubilisation by Surface-active Agents and its Application in Chemistry and Biological Sciences*. Chapman and Hall, London (1968).
15. Y. Mizushima and H. Suzuki, *Archs int. Pharmacodyn. Thér.* **157**, 115 (1965).
16. E. C. Ku and J. M. Wasvary, *Fedn Proc. Abstracts 57th Annual Meeting*, Vol. 32, p. 803 (1973).
17. H. J. Schaeffer, in *Drug Design* (Ed. E. J. Ariens), p. 129. Academic Press, New York (1971).
18. A. L. Willis, K. Comai, D. C. Kuhn and J. Paulsrud, *Prostaglandins* **8**, 509 (1974).
19. H. K. Mangold and T. Bezzegh, in *Clinical Biochemistry* (Eds. H. Ch. Curtius and M. Roth), p. 1033. Berlin, New York (1974).
20. S. Garattini and R. Paoletti, *Drugs Affecting Lipid Metabolism*, p. 454. Elsevier, Amsterdam (1961).