Biochemical Pharmacology, Vol. 30, No. 20, pp. 2811–2815, 1981.
Printed in Great Britain.

0006-2952/81/202811-05 $02.00/0

ANTIOXIDANTS INCREASE THE FORMATION OF 6-OXO-$PGF_{1\alpha}$ BY RAM SEMINAL VESICLE MICROSOMES*

JOHAN R. BEETENS†, MAGDA CLAEYS‡ and ARNOLD G. HERMAN
Faculty of Medicine, Division of Pharmacology, Universitaire Instelling Antwerpen, Universiteitsplein 1, B-2610 Wilrijk Belgium

(*Received* 10 *March* 1981; *accepted* 7 *May* 1981)

Abstract—The influence of antioxidants on the conversion of arachidonic acid and prostaglandin endoperoxide H_2 by ram seminal vesicle microsomes was investigated. The antioxidants ascorbic acid and propylgallate enhanced significantly the formation of 6-oxo-$PGF_{1\alpha}$. They shifted the pathway of the endoperoxide H_2 from the non-enzymic decomposition to the enzymic formation of prostacyclin. It is suggested that those antioxidants partially act through neutralisation of an oxidative species which is formed during the conversion of arachidonic acid. The other mechanisms through which they act remains to be elucidated.

Hydroperoxides of unsaturated fatty acids have been shown to inhibit the formation of prostacyclin (PGI_2) which is believed to prevent arterial thrombosis *in vivo* by protecting the arterial wall against deposition of platelets [1]. It has also been demonstrated that an important lipid peroxidation occurs during vitamin E deficiency, carcinogenesis and during hyperlipidaemia accompanying atherosclerosis [2]. Furthermore important amounts of lipid peroxides were found in advanced atherosclerotic plaques of human aortas [3]. On the other hand, several clinical and experimental studies reported the beneficial effect of antioxidants in diseases with haematological disorders [4–6].

These data suggest that there is a rational basis for evaluating the antioxidants on their ability to prevent lipid peroxidation and to increase PGI_2-formation, which could play a role in preventing the development of atherosclerotic and/or arterial thrombosis as earlier suggested [7]. In the present study, we therefore investigated the effects of antioxidants on the formation of PGI_2 by ram seminal vesicle (RSV) microsomes.

The choice of this enzyme system was based upon the recent observation by Cottee *et al.* [8] that RSV microsomes contain both the PG cyclo-oxygenase and the PGI-synthase. Furthermore, they found that 6-oxo-$PGF_{1\alpha}$, the non-enzymic degradation product of PGI_2, was the main product formed during incubations with low substrate/enzyme ratios and in the absence of cofactors (increasing the substrate concentrations resulted in a decreased formation of 6-oxo-$PGF_{1\alpha}$ and in an enhanced conversion to PGE_2 and PGD_2, the non-enzymic degradation products of PGH_2).

MATERIAL AND METHODS

Materials. Ram seminal vesicles were obtained from a local slaughter house. A particulate fraction containing cyclo-oxygenase activity was prepared as previously described [9] and stored as a lyophilised powder at −20°. Radioactive [1-^{14}C]arachidonic acid (56.5 mCi/mmole) was purchased from New England Nuclear (Boston, MA). Unlabelled arachidonic acid (AA), propylgallate and L-tryptophan were obtained from Sigma Chemical Co. (St. Louis, MO) and ascorbic acid from Roche Laboratories (Nutley, NJ). All prostaglandin standards ($PGF_{2\alpha}$, PGE_2, PGD_2, PGA_2 and 6-oxo-$PGF_{1\alpha}$) were a generous gift of Dr. J. Pike (Upjohn, Kalamazoo, MI). Thin layer chromatography (t.l.c.) was performed on silica plates $60F_{254}$ (Merck, Darmstadt, West Germany). All solvents used were of analytical grade (Merck).

Enzyme incubations. Incubations were carried out in a final volume of 1 ml Tris–HCl buffer (pH 7.5 at 25°, 100 mM). Each tube contained 10 mg of the freeze dried enzyme preparation (4.5–5.0 mg of protein), 250 ng [1-^{14}C]AA, various amounts of unlabelled AA and where applicable, different amounts of antioxidants. As the main goal of our experiments was the study of the effect of antioxidants on the formation of prostacyclin, no reduced glutathione was included in the incubation mixture, since Cottee *et al.* had shown that the formation of 6-oxo-$PGF_{1\alpha}$ was substantially suppressed in the presence of glutathione [8]. The incubations were started by adding the enzyme suspension to the AA substrate, were carried out for 20 min at 27° and terminated by acidification to pH 3.0 with HCl (0.5 N). The labelled reaction products were extracted into ethylacetate by vortex mixing (2 × 2 vol.). The organic layers were combined, dried under nitrogen, redissolved in 50 μl chloroform:methanol (2:1) and applied on

* Part of this work has been presented at the meeting of the British Pharmacological Society, London, December 1979.

† I.W.O.N.L. postgraduate student (Grant 78183).

‡ Research associate of the Belgian National Fonds voor Wetenschappelijk Onderzoek.

to a t.l.c. plate, together with authentic standards (6-oxo-$PGF_{1\alpha}$, $PGF_{2\alpha}$, PGE_2, PGD_2 and PGA_2; 2 μg of each). The plate was developed with the organic phase of 2-2-4-trimethylpentane/ethylacetate/acetic acid/water 5:11:2:10). Radioactive zones were located by radiochromatogram-scanning and the standards detected with phosphomolybdic acid. The zones corresponding to the different metabolites were scraped off and the radioactivity determined by conventional liquid scintillation counting. The amount of the metabolites formed was expressed as the percentage of the total amount of radioactivity recovered. In some experiments, the absolute amount of the metabolites was calculated from these percentages. For a few samples, the absolute amount of 6-oxo-$PGF_{1\alpha}$ was confirmed by radioimmunoassay. For the statistical evaluation, the control experiment was taken as 100% and the values obtained in the experiments with the antioxidants were expressed as the per cent of the values found in the control experiment. The paired Student's-*t* test and the Wilcoxon test for paired observations were used. For the evaluation of the differences between the several agents or incubation methods the Sign test or Rang sign test were used.

RESULTS

The enzymic system. In the absence of antioxidants, similar results as those obtained by Cottee *et al.* were observed (see Fig. 1). At a low concentration of AA (0.25 and 2 μg/ml, 0.8 and 6.6 μM) about 70% of the added amount of AA was converted to 6-oxo-$PGF_{1\alpha}$. As the substrate concentration was increased, the percentual yield of 6-oxo-$PGF_{1\alpha}$ was diminished and other metabolites a.o. PGE_2 and PGD_2 were predominantly formed. However, the absolute amount of 6-oxo-$PGF_{1\alpha}$ did not decrease with increasing AA concentrations (Fig. 2) but reached a plateau value at a relative low substrate concentration (5 μg AA/ml, 16.5 μM). From this concentration on, no influence of the substrate on the synthesis of 6-oxo-$PGF_{1\alpha}$ was observed. In contrast, there was a continuous linear relationship between the amount of AA and the formation of PGE_2 and PGD_2, indicating a non-enzymic breakdown of the PG endoperoxide H_2 (PGH_2).

Influence of antioxidants. Ascorbic acid (vitamin C) in concentrations up to 100 μg/ml (570 μM) did not affect the overall conversion of AA, which was always complete. No influence on any of the AA metabolites formed was seen with 3 and 6 μg vitamin C per ml of incubation medium (17 and 34 μM). From 12 μg vitamin C on (68 μM) a dose dependent increase in 6-oxo-$PGF_{1\alpha}$ production was observed (Table 1). Concomitantly, the formation of PGE_2 and PGD_2 was significantly diminished. Representive radiochromatograms of these experiments are shown in Fig. 3. This figure also shows the presence of an unknown peak (X, R_f value:0.58) in the control experiment during the incubations with high amounts of AA; this product was absent in the corresponding experiments in which vitamin C was included.

$PGF_{2\alpha}$, which was generally formed in small amounts was not affected by small concentrations of vitamin C. However, from 36 μg vitamin C on, a highly significant increase in $PGF_{2\alpha}$ formation was observed (Table 2).

Incubations were also carried out with the antioxidant propylgallate in concentrations ranging from 0.31 to 10 μg/ml (1.5–47 μM). With 0.65–5.0 μg/ml similar results as with vitamin C were obtained: increased production of 6-oxo-$PGF_{1\alpha}$, decreased formation of PGE_2, PGD_2 and metabolite X, and at the higher concentrations of propylgallate increased formation of $PGF_{2\alpha}$. High concentrations of propylgallate (7.5 and 10 μg/ml) however did affect the overall conversion of AA and therefore were not further investigated.

Influence of L-*tryptophan.* Recently, it was reported that 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid (15-HPETE) exerts its inhibitory action on the PGI-synthase by the production of a destructive oxidative species, probably an OH-radical or a

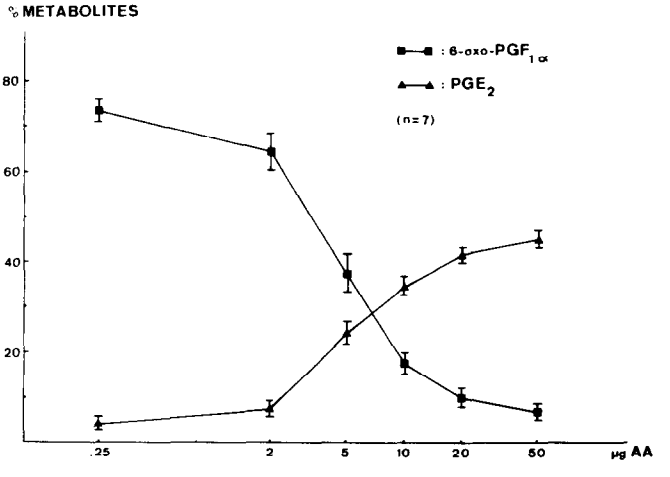

Fig. 1. Procentual formation of 6-oxo-$PGF_{1\alpha}$ and PGE_2 by ram seminal vesicle microsomes incubated with arachidonic acid (AA).

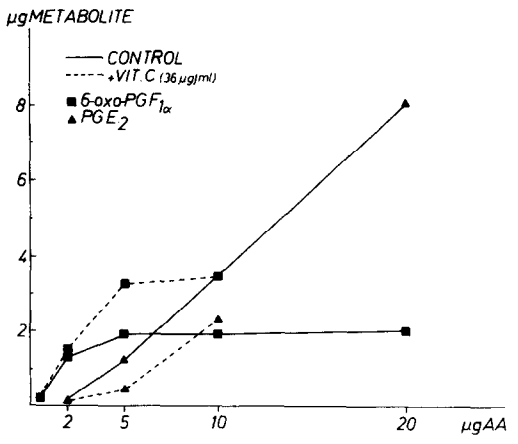

Fig. 2. Effect of increasing concentrations of AA on the formation of 6-oxo-PGF$_{1\alpha}$ and PGE$_2$, and the influence of vitamin C.

species of similar reactivity [10]. Furthermore, tryptophan protected the PGI-synthase activity against 15-HPETE [10]. In order to study whether the action of vitamin C could be explained in a similar way, RSV microsomes were incubated with different concentrations of L-tryptophan (200, 400 and 1000 μg/ml; 1, 2 and 5 mM). These concentrations have been reported to give 95% protection against the inhibitory activity of various amounts of 15-HPETE [10].

In our system, 2 and 5 mM tryptophan increased significantly the production of 6-oxo-PGF$_{1\alpha}$ (Rang sign test, $P < 0.05$, $n = 8$). In contrast with vitamin C, it did not increase PGF$_{2\alpha}$ formation at higher concentrations. However the increase in 6-oxo-PGF$_{1\alpha}$ formation with both concentrations of tryptophan used, was less than obtained with 24 μg vitamin C when incubated under the same conditions (Sign test, $P < 0.05$, $n = 8$).

Metabolism of PGH$_2$. In order to find out in which step of PGI$_2$ biosynthesis vitamin C exerts its effect, experiments were carried out with PGH$_2$. Vitamin C could act through the neutralisation of oxidative species (O_x) which have been reported to be generated during PGH$_2$ formation [11] and which are particularly destructive to PGI-synthase [12].

[^{14}C]PGH$_2$ (1.5 mCi/mole) was prepared using RSV microsomes and following the procedures described by Nugteren and Hazelhof [13]. Optimal formation of 6-oxo-PGF$_{1\alpha}$ was obtained with 4 and 10 μg/ml (11.5 and 28.5 μM) of PGH$_2$. Therefore RSV microsomes were incubated with these concentrations of PGH$_2$ in the absence or presence of various amounts of vitamin C (12–100 μg/ml, 60–560 μM) and under the same conditions as those used for the experiments with AA.

From 24 μg/ml of vitamin C on, a small increase in the formation of 6-oxo-PGF$_{1\alpha}$ was observed. Although this increase was statistically significant it was smaller than the increase observed with vitamin C during [^{14}C]AA incubation (Table 3). However, in the experiments described above, it could not be ruled out that endogenous AA, present in the microsomes or released from their phospholipid fraction, was converted to PGG$_2$ and PGH$_2$ which could still give rise to the production of a destructive oxygen species, thereby leading to a partial inhibition of PGI-synthase. This could explain the small increase of 6-oxo-PGF$_{1\alpha}$ during the incubation of PGH$_2$ in the presence of vitamin C.

Therefore, PGH$_2$ experiments were also carried out in the presence of a high amount of indomethacin (2×10^{-5}M) which would block the conversion of the endogenous AA.

In the absence of vitamin C, indomethacin increased slightly but not significantly the conversion of PGH$_2$ to 6-oxo-PGF$_{1\alpha}$. However indomethacin was not able to augment further the increased con-

Table 1. Formation of 6-oxo-PGF$_{1\alpha}$ by ram seminal vesicle microsomes incubated with AA

AA (μg/ml)	Ascorbic acid (μg/ml)					
	0	12	24	36	72	100
2	100	108 + 3.7 ▲	112.4 + 4.2 ●	123 ± 10 ▲	130 + 9 ■	185 ± 56
5	100	142 ± 12.5 ●	160 ± 14.2 ●	154 ± 12 ●	152.5 ± 28.5	175 ± 39
10	100	145 ± 36	162 ± 14.4 ●	161.5 ± 15 ■	165 ± 15 ●	173 ± 22.5 ●

Significance: ▲$P < 0.05$, *$P < 0.025$, ●$P < 0.01$, ■$P < 0.005$ (Student's-t test). The results are expressed as a percentage of the amount of 6-oxo-PGF$_{1\alpha}$ formed in the absence of antioxidant; mean ± S.E.M; $n \geq 6$.

Table 2. Formation of PGF$_{2\alpha}$ by ram seminal vesicle microsomes incubated with AA

AA (μg/ml)	Ascorbic acid (μg/ml)					
	0	12	24	36	72	100
2	100	94 ± 13	110 ± 21	117 ± 20	167 ± 25 ●	137 ± 11 ●
5	100	143 ± 31	152 ± 37	223 ± 37 ●	322 ± 41 ■	324 ± 47 ■
10	100	144 ± 36	160 ± 40	308 ± 40 ■	446 ± 51 ■	456 ± 51 ■

Significance: ▲$P < 0.05$, *$P < 0.025$, ●$P < 0.01$, ■$P < 0.005$ (Student's-t test). The results are expressed as a percentage of the amount of PGF$_{2\alpha}$ formed in the absence of antioxidant; mean ± S.E.M. $n \geq 6$.

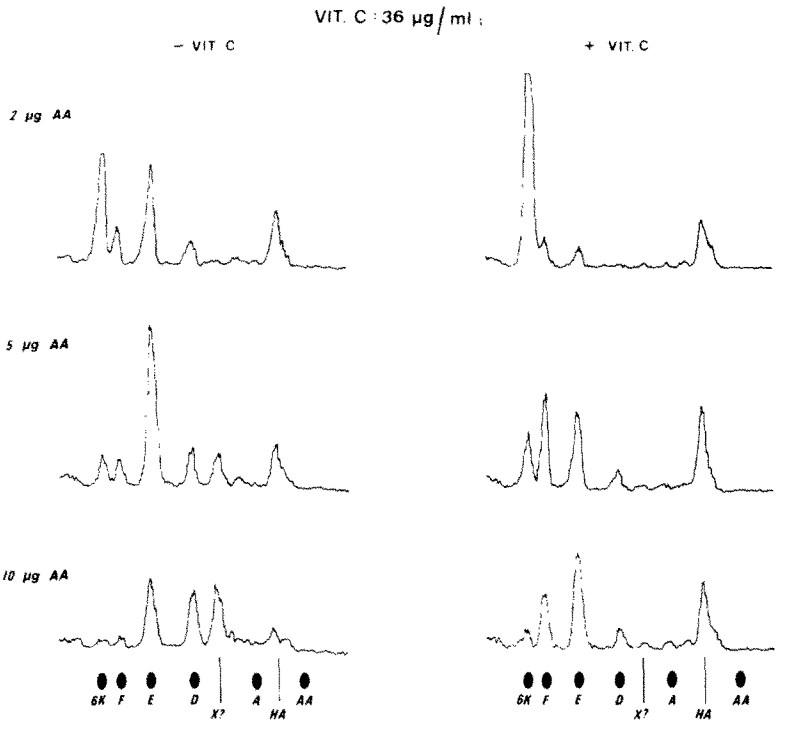

Fig. 3. Radiochromatograms of incubations of AA with RSV microsomes. On the left side, the control experiments; on the right side, the experiments in which vitamin C (36 µg/ml) was co-incubated. 6 K, 6-oxo-$PGF_{1\alpha}$; F, $PGF_{2\alpha}$; E, PGE_2; D, PGD_2; X?, unknown; A, PGA_2; HA, hydroxy-fatty acids; AA, arachidonic acid.

version of PGH_2 which was observed when vitamin C was present in the incubation mixture.

DISCUSSION

Cottee *et al.* [8] reported a decreased formation of 6-oxo-$PGF_{1\alpha}$ with increasing AA concentrations. Our experiments confirm that the percentual formation of 6-oxo-$PGF_{1\alpha}$ is decreasing with increasing amounts of AA. However, the absolute amounts of 6-oxo-$PGF_{1\alpha}$ formed reach a plateau value which was not further influenced by increasing the substrate concentration.

The antioxidants, vitamin C and propylgallate, significantly enhance the formation of 6-oxo-$PGF_{1\alpha}$. The pathway of the PGH_2 metabolism is clearly shifted from its non-enzymic decomposition to the enzymic formation of PGI_2. This stimulation of PGI_2

Table 3. Formation of 6-oxo $PGF_{1\alpha}$ by ram seminal vesicle microsomes incubated with PGH_2 and with AA. The results are expressed as a percentage of the amount of 6-oxo-$PGF_{1\alpha}$ formed in the absence of antioxidant, mean ± S.E.M. ($n = 6$).

Substrate (µg/ml)		Ascorbic acid (µg/ml)				
		0	12	24	36	100
PGH_2 :	4	100	103 ± 4 ■	109 ± 3 *■	119 ± 4 *■	128 ± 8 *■
	10	100	103 ± 3 ▲	109 ± 3 *▲	107 ± 4 *▲	112 ± 2,5 *▲
AA :	4	100	117 ± 6 *■	125 ± 4 *■	132 ± 4 *■	145 ± 12 *■
	10	100	130 ± 12 *▲	134 ± 10 *▲	140 ± 11 *▲	155 ± 6 *▲

*$P < 0.05$ = significantly different from control (Sign test), ■ and ▲ $P < 0.05$ = significantly different from corresponding value in both substrate groups (Rang-sign test, Wilcoxon). In both substrate groups the amount of 6-oxo-$PFG_{1\alpha}$ obtained in the presence of various concentrations of ascorbic acid was compared with that obtained in the absence of the antioxidant. For each concentration of ascorbic acid the amount of 6-oxo-$PGF_{1\alpha}$ derived from PGH_2 was compared with the corresponding amount obtained from AA.

formation can be explained by various mechanisms. Recently, Ravikumar *et al.* reported that the incubation medium of RSV microsomes with AA contains 15-hydroxy-5,8,11,13-eicosatetraenoic acid (15-HETE) [14]. This finding implies that its precursor, 15-HPETE, which is known to be destructive to PGI-synthase [15], was also generated. Furthermore, it was reported by Weiss *et al.* [10] that L-tryptophan, a potent $OH^{\cdot}$ radical scavenger, protects PGI-synthase from the inhibitory activity of 15-HPETE. The enhanced formation of 6-oxo-$PGF_{1\alpha}$, observed in our experiments in the presence of L-tryptophan, indicate a possible involvement of an $OH^{\cdot}$ radical in the enzyme system used. Consequently, the stimulating effect of vitamin C could be the result of its action as an $OH^{\cdot}$ scavenger. However, since the increased formation of 6-oxo-$PGF_{1\alpha}$ in the presence of L-tryptophan was much less pronounced than the one observed in the presence of vitamin C or propylgallate, the effect of the latter cannot completely be attributed to their $OH^{\cdot}$ scavenger-like activity.

Although it cannot be ruled out that vitamin C and propylgallate interfere directly with the formation of 15-HPETE, it can more easily be rationalised that these substances neutralise the oxidative species, which is generated by 11-HPETE and which can be formed by partial PG-cyclooxygenase action [16]. It is much more likely however that vitamin C and propylgallate neutralise the oxidative species, which is formed during reduction of PGG_2 to PGH_2 and for which it has been reported by Ham *et al.* that it is particularly destructive to PGI-synthase [12]. The experiments, in which PGH_2 was incubated in the presence of vitamin C, still showed a small but significant enhancement in the formation of 6-oxo-$PGF_{1\alpha}$, which was not altered by the presence of indomethacin in the incubation mixture. These results indicate that vitamin C acts primarily through the neutralisation of the oxidative species, which is formed during the conversion of AA. However, the results also demonstrate that this effect cannot explain the entire mechanism through which vitamin C stimulates the formation of PGI_2.

At the higher concentrations of vitamin C and propylgallate, the formation of $PGF_{2\alpha}$ is increased. This is probably due to a more direct reducing effect of the antioxidants on PGH_2.

During the incubations with high amounts AA, in the absence of antioxidants, an unknown peak X, with R_f value 0.58, was observed. A substance with comparable R_f value has been identified as 15-oxo-PGE_2 and its formation has been rationalised through a non-enzymic decomposition of 15-hydroperoxy-PGE_2 or PGG_2 in the absence of a reducing agent [13, 17]. Our observation that X is not formed when the antioxidants are coincubated therefore suggests that X is most likely 15-oxo-PGE_2.

The experiments described clearly demonstrate the influence of the antioxidants, vitamin C and propylgallate, on the metabolism of AA in RSV microsomes. Although the stimulatory activity of these antioxidants on the production of 6-oxo-$PGF_{1\alpha}$ can be partially explained through a combination of the following mechanisms i.e. (1) scavenging or neutralisation of an $OH^{\cdot}$ radical or other oxidative species destructive to the PGI-synthase and which are produced during the conversion of AA to PGH_2 and/or the conversion of 15-HPETE and 11-HPETE to 15-HETE and 11-HETE respectively, or (2) interference with the formation of 15-HPETE, other effects could still be involved.

Indeed, our experiments do not rule out the possibility that these antioxidants, for example, stimulate directly the PGI-synthase or prevent its destruction. Whether vitamin C and propylgallate have a stimulatory effect on the enzymatic formation of PGI_2 in the microsomal fraction of other tissues and in whole tissues, e.g. the vascular wall, remains to be established.

REFERENCES

1. S. Moncada, R. Gryglewski, S. Bunting and J. R. Vane, *Nature, Lond.* **263**, 663 (1976).
2. T. F. Slater, in *Free Radical Mechanism in Tissue Injury.* Pion Ltd. London (1972).
3. W. A. Harland, J. D. Gilbert and C. J. W. Brooks, *Biochim. biophys. Acta,* **316**, 378 (1973).
4. I. Nafstad, *Tox. Lett.* **2**, 313 (1978).
5. C. R. Spittle and M. R. C. Path, *Am. Heart J.* **88**, 387 (1974).
6. M. B. Donati, G. Remuzzi, D. Marchesi, R. Misiani, M. Livio, G. Mecca and G. de Gaetano, *Abstracts of Int. Symp. on Endothelium, Platelets and Prostaglandins.* Bergamo, Sept. 1979.
7. R. J. Gryglewski, S. Bunting, S. Moncada, R. J. Flower and J. R. Vane, *Prostaglandins* **12**, 685 (1976).
8. F. Cottee, R. J. Flower, S. Moncada, J. A. Salmon and J. R. Vane, *Prostaglandins* **14**, 413 (1977).
9. C. Takeguchi, E. Kohno and C. J. Sih, *Biochemistry* **10**, 2372 (1971).
10. S. J. Weiss, J. Turk and P. Needleman, *Blood* **53**, 1191 (1979).
11. R. W. Egan, J. Paxton and F. A. Kuehl Jr., *J. biol. Chem.* **251**, 7329 (1976).
12. E. A. Ham, R. W. Egan, D. D. Saderman, P. H. Gale and F. A. Kuehl Jr., *J. biol. Chem.* **254**, 2191 (1979).
13. D. H. Nugteren and E. Hazelhof, *Biochim. biophys. Acta* **326**, 448 (1973).
14. P. R. Ravikumar, J. K. Pat, N. J. Zmijewski and C. J. Sih, *J. Pharm. Sci.* **68**, 1302 (1979).
15. S. Moncada, R. J. Gryglewski, S. Bunting and J. R. Vane, *Prostaglandins* **12**, 715 (1976).
16. M. E. Hemler, C. G. Crawford and W. E. M. Lands, *Biochemistry* **17**, 1772 (1978).
17. D. H. Nugteren, R. K. Beerthuis and D. A. Van Dorp, *Recl Trav. chim. Pays-Bas* **85**, 405 (1966).