TABLE IV

Compound	Salt	Yield, %	Mp, °C	Formula	Analyses
1-Methyl-4,5-diphenylpiperazine (**3**)	2HCl	85	224–226	$C_{17}H_{22}C_2N_2$	C, H, N
2-Methyl-1,3,4,13b-tetrahydro-2H-pyrazino[1,2-*f*]-phenanthridine (**4**)	HCl	92	236–240	$C_{17}H_{19}ClN_2$	C, H, N, Cl
2-Methyl-1,2,3,4,10,14b-hexahydropyrazino[1,2-*f*]-morphanthridine (**5**)	HCl	90	282–284	$C_{18}H_{21}ClN_2$	C, H, N, Cl
2-Methyl-1,3,4,10,11,15b-hexahydro-2H-dibenz[*c*,*g*]-pyrazino[1,2-*a*]azocine (**6**)	HCl	43	305–310	$C_{19}H_{23}ClN_2$	C, H, N, Cl

heated during 45 min from room temperature to 140°. The temperature was then raised to 180° in 15 min and kept at this level for 0.5 hr. During the reaction about 50 ml of EtOH distilled off. The reaction mixture was cooled and treated with 200 ml of C_6H_6. The crystals were filtered, washed with Et_2O, and dried *in vacuo* to give the diketopiperazino compounds listed in Table III.

Diborane Reduction of the Substituted Diketopiperazines 11, 15, 21, and 29 to the Piperazino Compounds 3–6.—To a suspension of 120 g (3.2 moles) of $NaBH_4$ in 350 ml of dry THF was added dropwise a solution of 520 ml of BF_3 etherate during 2 hr. The generated B_2H_6 was introduced directly with stirring into a suspension of 100 g of the diketopiperazino compound. The entire manipulation was carried out under N_2. The mixture was then refluxed for 1 hr. Excess B_2H_6 was decomposed by adding slowly 350 ml of 96% EtOH, and the solution was evaporated to dryness. The vitreous residue was dissolved in 1800 ml of 18% aqueous HCl and heated on a steam bath for 1 hr, cooled, made alkaline with 30% NaOH, and extracted with CH_2Cl_2. The extract was washed with H_2O, dried (Na_2SO_4), and evaporated to dryness to give 90–95% of the oily piperazino compound. The product was converted to its hydrochloride with alcoholic HCl and recrystallized from EtOH. In this way the compounds listed in Table IV were obtained.

Effect of Eight Prostaglandins on Platelet Aggregation[1]

N. CHANDRA SEKHAR[2]

Cardiovascular Diseases Research, The Upjohn Company, Kalamazoo, Michigan 49001

Received April 14, 1969

Eight prostaglandins, PGE_1, PGE_2, PGA_1, PGA_2, $PGF_{1\alpha}$, $PGF_{2\alpha}$, $PGF_{1\beta}$, and $PGF_{2\beta}$, were tested for their effect on platelet aggregation–adhesion induced by ADP, thrombin, and collagen. All compounds inhibited aggregation in platelet-rich rat plasma and human plasma to varying degrees. PGE_1 was the most active compound in the group. In addition, PGE_1 showed very potent thrombolytic effect against ADP-induced platelet thrombi *in vitro*. A single intravenous injection of 3 mg of PGE_1/kg in rats inhibited platelet aggregation in blood samples withdrawn from animals 30 min following the injection. Platelet aggregation was also inhibited significantly in rats given infusions of PGE_1 at 1.8 mg/kg per day for 30 days.

Prostaglandins are powerful vasoactive compounds which occur in human seminal plasma, sheep seminal vesicles, and other tissues. Born and coworkers[3] have reported that a number of vasoactive compounds show a corresponding ability to affect platelet behavior. Since prostaglandin E_1 (PGE_1) is known to be a potent vasodilator, it was of interest to investigate the influence of PGE_1 and other prostaglandins on platelet aggregation. Kloeze[4] first reported that PGE_1 inhibits ADP-induced aggregation as well as glass adhesion of platelets, whereas PGE_2 accelerates platelet aggregation. Weeks, Sekhar, and DuCharme[5] have reported the comparative activity of prostaglandins E_1, A_1, E_2, and A_2 on aggregation of rabbit platelets. The present paper deals with the influence of eight structurally related prostaglandins on adenosine diphosphate (ADP), thrombin (recalcified plasma), and collagen induced aggregation of human and rat platelets.

Experimental Section

Prostaglandins.—The following prostaglandins have been studied: 11α,15(*S*)-dihydroxy-9-oxo-13-*trans*-prostenoic acid (PGE_1), 11α,15(*S*)-dihydroxy-9-oxo-5-*cis*,13-*trans*-prostadienoic acid (PGE_2), 15(*S*)-hydroxy-9-oxo-10,13-*trans*-prostadienoic acid (PGA_1), 15(*S*)-hydroxy-9-oxo-5-*cis*-10,13-*trans*-prostadienoic acid (PGA_2), 9α,11α,15(*S*)-trihydroxy-13-*trans*-prostenoic acid ($PGF_{1\alpha}$), 9α,11α,15(*S*)-trihydroxy-5-*cis*,13-*trans*-prostadienoic acid ($PGF_{2\alpha}$), 9β,11α,15(*S*)-trihydroxy-13-*trans*-prostenoic acid ($PGF_{1\beta}$), 9β,11α,15(*S*)-trihydroxy-5-*cis*,13-*trans*-prostadienoic acid ($PGF_{2\beta}$). The purity of all prostaglandins was checked by tlc.

Inhibition of Platelet Aggregation *in Vitro*.—Chandler's revolving plastic loop technique as modified by Silver[6] was used for the study of platelet aggregation and thrombus formation. Blood from the abdominal aorta of normal male rats (Sprague–Dawley, Spartan strain) was drawn into a plastic syringe containing sodium citrate solution (1 part of 3.8% sodium citrate solution to 9 parts of blood), thoroughly mixed by gentle tilting of the syringe, and centrifuged at 1200 rpm for 10 min. The supernatant platelet-rich plasma was collected by siliconized Dispo pipets. This platelet-rich rat plasma (PRRP, 0.8 ml) was transferred into a plastic loop (Transflex tubing No. 8, 3M Company) by a plastic syringe and 0.1 ml of 0.85% saline was added. The loop was closed with a short segment of another plastic tubing (Transflex tubing No. 6), mounted on a disk, and rotated at 12 rpm for 1 min. One tenth milliliter of 0.25 *M* $CaCl_2$ solution was injected through the loop onto the plasma layer; four stop watches and the loop were started simultaneously. Four successive end points were timed: aggregation of

(1) (a) A preliminary report of this work was presented orally at the Third Platelet Conference, Oak Ridge, Tenn., June 1967; (b) N. Chandrasekhar, *Blood*, **30**, 554 (1967).

(2) Formerly N.Chandrasekhar.

(3) G. V. R. Born, R. J. Haslam, N. Goldman, and R. D. Lowe, *Nature*, **205**, 678 (1965).

(4) J. Kloeze, Nobel Symposium 2, Prostaglandins, S. Bergström and B. Samuelsson, Ed., Almquist and Wiksell, Stockholm, 1967, p 241.

(5) J. R. Weeks, N. C. Sekhar, and D. W. DuCharme, *J. Pharm. Pharmacol.*, **21**, 103 (1969).

(6) M. J. Silver, *Am. J. Physiol.*, **209**, 1128 (1965).

platelets visible to the naked eye (VA), snowstorm-like platelet clumping (SS), formation of a well-packed platelet disk (head) at one end of the plasma surface (PH), and the formation of a characteristic thrombus with white platelet head and a thin fibrin tail (TF). To observe the effects of various prostaglandins on platelet behavior, 0.1 ml of the test solutions (instead of saline) was mixed with PRRP.

The effect of prostaglandins on formation of VA, SS, and PH in freshly obtained platelet-rich human plasma (PRHP, obtained by centrifuging freshly collected citrated human blood at 800 rpm for 10 min) was studied using 0.1 ml of ADP solution (200 μg of ADP dissolved in 1 ml of Ringer's solution) in place of $CaCl_2$ solution as the aggregation inducer. The effect of prostaglandins on adhesion and aggregation of platelets induced by collagen was studied using an Aggregometer (Bryston Manufacturing Co., Scarborough, Canada). The Aggregometer measures the decrease in optical density as platelet aggregation occurs progressively in the plasma sample tested. To obtain control values, 0.1 ml of a collagen (Sigma Chemical Co.) suspension (in Tyrode solution without calcium) was added to 1.1 ml of a plasma–saline mixture placed in Aggregometer cuvettes and the adhesion–aggregation reaction was recorded on a Bausch and Lomb VOM 6 recorder. The different prostaglandin solutions (0.1 ml), instead of saline, were incubated with platelet-rich plasma and the recorded curve was followed after addition of collagen. The effect of PGE_1 and PGE_2 on the ADP-induced aggregation in PRRP was also studied at various concentrations of ADP (0.3, 0.5, 1.0, and 20 μg/ml) using the Aggregometer.

Thrombolytic Effect.—In order to study the thrombolytic effect of prostaglandins, 0.1 ml of saline was injected into the plastic loop after an ADP-induced platelet head was formed in PRHP. The time required for the platelet head to lyse was noted as the control time. The lysis time after injecting prostaglandin solutions of various concentrations (instead of saline) was then determined. To observe the effect of prostaglandins on dispersing collagen-induced platelet aggregation, the compounds were injected into the Aggregometer cuvette after the recorder curve had reached a maximum. The subsequent downward slope of the curve indicated the degree of dispersion of platelet aggregates.

Intravenous Effect.—To determine whether the prostaglandin most active *in vitro* (PGE_1) was active *in vivo*, four different doses (0.5, 1.0, 2.0, and 3.0 mg/kg) were administered intravenously (tail vein) to rats. Animals were sacrificed at 5, 10, 15, 30, 60, or 120 min after injection to obtain platelet-rich plasma and the different end points (VA, SS, PH, and TF) were determined in the revolving loop using $CaCl_2$ (similar to thrombin) to induce aggregation. Inhibition of collagen-induced aggregation was estimated by comparing the heights of Aggregometer curves of experimental groups with that of control injected with saline.

To evaluate the long-term effect of PGE_1, mature Upjohn Sprague–Dawley rats (males and females, two each in the control group and four each in the experimental group) were prepared with chronic indwelling venous cannulas[7] and fitted with a saddle and cannula feed-through (No. 1601, Lehigh Valley Electronics Co., Fogelsville, Pa.), which permitted relatively unrestrained movement. PGE_1 was given at 1.8 mg/kg per day, divided among ten equally spaced 5-min infusions. A similar group of rats was given saline. After 30 days, prothrombin times and platelet aggregation parameters were determined.

Oral Effect.—Groups of three rats were given 10 mg of PGE_1 or saline/kg and sacrificed after 0.5, 1.0, 2.0, 4.0, or 6.0 hr following dosing, to obtain blood samples for determination of platelet aggregation parameters as described above.

Results

Seven of the eight prostaglandins studied inhibited rat platelet aggregation at concentrations of 100 μg/ml or lower (Table I). PGE_1 was the most active compound in the group, inhibiting aggregation at a minimal concentration of 0.05 μg/ml. $PGF_{2\beta}$ did not inhibit VA, SS, or PH in PRRP; instead it accelerated thrombus formation. $PGF_{2\alpha}$, PGA_2, and $PGF_{1\beta}$ also showed a similar acceleration of thrombus formation.

PGE_2 inhibited thrombin-induced aggregation (aggregation in recalcified plasma) in PRRP at 12.5

(7) J. R. Weeks and J. D. Davis, *J. Appl. Physiol.*, **19**, 540 (1964).

TABLE I
INHIBITION OF AGGREGATION IN PLATELET-RICH RAT PLASMA (PRRP) BY PROSTAGLANDINS *in Vitro*[a]

Compd, min effective conen (μg/ml)	Aggregation in control PRRP[b]			Aggregation in prostaglandin-incubated PRRP[b]			
	VA	SS	PH	TF	VA	SS	PH	TF
PGE_1, 0.05	16.7 ± 0.5	23.3 ± 0.9	29.7 ± 1.6	193.1 ± 9.8	168.9 ± 14.9 (+911%)[c]	182.3 ± 7.9 (+682%)	183.1 ± 7.6 (+517%)	184.7 ± 7.2 (−4%)
PGE_2, 12.5	18.0 ± 0.7	24.1 ± 0.8	31.3 ± 1.5	180.8 ± 9.6	25.5 ± 1.3 (+42%)[c]	102.2 ± 16.3 (+324%)[c]	137.6 ± 12.6 (+340%)[c]	183.2 ± 11.6 (+1%)
PGA, 30.0	17.3 ± 0.7	22.8 ± 0.7	29.2 ± 1.4	172.5 ± 9.9	28.3 ± 2.9 (+64%)[c]	113.5 ± 14.9 (+398%)[c]	132.9 ± 11.2 (+355%)[c]	173.4 ± 9.4 (+0.5%)
PGA_2, 70.0	18.3 ± 0.7	23.9 ± 0.9	29.5 ± 1.0	195.9 ± 8.4	27.4 ± 1.5 (+50%)[c]	102.8 ± 14.3 (+330%)[c]	135.2 ± 8.0 (+358%)[c]	165.9 ± 5.4 (−15%)[c]
$PGF_{1\alpha}$, 50.0	17.3 ± 1.0	23.9 ± 1.3	31.6 ± 1.9	203.3 ± 8.9	31.4 ± 16.2 (+197%)[c]	152.7 ± 15.4 (+539%)[c]	158.3 ± 15.4 (+401%)[c]	185.9 ± 6.9 (−9%)
$PGF_{2\alpha}$, 100.0	17.4 ± 0.7	23.1 ± 1.0	34.3 ± 2.1	198.5 ± 8.1	50.1 ± 18.7 (+188%)[c]	112.9 ± 11.3 (+389%)[c]	129.6 ± 6.9 (+278%)[c]	166.1 ± 3.5 (−16%)[c]
$PGF_{1\beta}$, 100.0	17.2 ± 0.5	22.7 ± 0.5	29.7 ± 1.9	183.6 ± 6.6	30.9 ± 3.4 (+80%)[c]	112.2 ± 12.0 (+394%)[c]	132.4 ± 7.0 (+346%)[c]	152.4 ± 5.4 (−17%)[c]
$PGF_{2\beta}$, 100.0	16.4 ± 0.7	22.5 ± 1.3	30.3 ± 2.1	170.5 ± 5.0	16.6 ± 0.6 (+1%)	22.8 ± 0.7 (+1%)	33.4 ± 3.6 (+10%)	143.0 ± 4.0 (−16%)[c]

[a] Ten samples for each compound; control and experimental values determined on portions of the same PRRP. [b] Aggregation in recalcified plasma is referred to, as thrombin-induced aggregation; time in seconds expressed as mean ± standard error. [c] Statistically significant, $p < 0.05$ by paired t test.

μg/ml and at higher concentrations, but showed no effect at lower concentrations in the revolving plastic loop. But PGE_2 in the concentration range of 3.0–0.1 μg/ml potentiated ADP-induced aggregation slightly (final concentrations of ADP, 0.5 μg/ml) in PRRP in the Aggregometer. PGE_2 inhibited aggregation of PRRP at 10 μg/ml but was ineffective at concentrations lower than 0.1 μg/ml in the same experimental system. The potentiating effect of PGE_2 on ADP-induced aggregation of rat platelets was seen only when the ADP concentration was in the range of 0.5–1.0 μg/ml; at 0.3 μg of ADP/ml or at 20.0 μg/ml, the potentiating effect was not observed. PGE_2 in the concentration range of 100–0.01 μg/ml did not show any potentiating effect of ADP (0.5 μg/ml)-induced aggregation in PRHP.

Inhibitory activity of prostaglandins on aggregation of human platelets (Table II) was almost parallel to that observed with rat platelets except that $PGF_{2\beta}$ was active in PRHP at a concentration of 100 μg/ml. Prostaglandins appeared to be most active against ADP-induced human platelet aggregation; many of the compounds totally inhibited SS and PH phenomena. In the case of human platelets also, PGE_1 was the most active compound, inhibiting platelet aggregation at a final concentration of 0.05 μg/ml.

TABLE II
INHIBITION OF HUMAN PLATELET AGGREGATION BY PROSTAGLANDINS *in Vitro*[a]

Compd, min effective concn (μg/ml)	Platelet aggregation parameters[b] VA	SS	PH
Control, 0	15.8 ± 0.3	21.4 ± 0.4	25.8 ± 0.6
PGE_1, 0.05	33.2 ± 3.0 (+110%)[c]	*d*	*d*
PGE_2, 20.0	23.8 ± 1.9 (+51%)	41.8 ± 3.3 (+95%)[c]	53.2 ± 8.0 (+106%)[c]
PGA_1, 30.0	30.5 ± 4.5 (+93%)[c]	>43[e]	*d*
PGA_2, 70.0	23.7 ± 2.4 (+50%)	45.3 ± 8.5 (+113%)[c]	45.8 ± 5.0 (+78%)[c]
$PGF_{1\alpha}$, 70.0	25.2 ± 2.2 (+60%)	49.8 ± 7.5 (+133%)[c]	>42[e]
$PGF_{1\beta}$, 100.0	33.2 + 4.6 (+106%)[c]	>48[e]	>70[e]
$PGF_{2\beta}$, 100.0	32.5 ± 6.3 (+106%)[c]	>42[e]	*d*
$PGF_{2\alpha}$, 100.0	23.8 ± 1.9 (+51%)	41.8 ± 3.3 (+95%)[c]	53.2 ± 8.0 (+106%)[c]

[a] Twelve samples for controls and six samples each for prostaglandins. [b] ADP-induced aggregation; time in seconds expressed as mean ± standard error. [c] Statistically significant, $p < 0.05$ by Student's t test. [d] The reaction did not occur. [e] The reaction occurred only in some of the samples; the lowest value obtained in the group is shown.

Since PGE_1 was the most potent inhibitor of ADP- and thrombin-induced platelet aggregation, the effect of the compound against collagen-induced adhesion was examined. The results presented in Figure 1 show that even a low concentration of PGE_1 (0.1 μg/ml) inhibits collagen-induced adhesion and aggregation of PRHP. Similar experiments with PRRP (Figure 2) showed that PGE_1 was equally active against collagen-induced rat platelet aggregation at 0.1 μg/ml. The inhibitory action of PGE_1 was concentration dependent in the range 0.05–0.2 μg/ml. Concentrations of 0.2 μg/ml completely blocked the occurrence of all aggregation parameters; concentrations above 0.2 μg/ml could not affect the reactions further. Concentrations lower than 0.05 μg/ml gave variable to no effect on platelet aggregation of both rat and human platelet-rich plasmas.

Experiments to determine whether PGE_1 would affect already formed platelet thrombi in the revolving plastic loop showed that even low concentrations of the compound would break up and completely lyse ADP-induced human platelet thrombi within a few minutes. Up to 0.1 μg/ml, the total lysis time was inversely proportional to the concentration of PGE_1 added. Higher concentrations of PGE_1 did not shorten the lysis time further (Table III). Similar thrombolytic activity of PGE_1 was also observed on collagen-induced platelet aggregates in the Aggregometer (Figure 2).

TABLE III
EFFECT OF PGE_1 ON LYSIS OF ADP-INDUCED HUMAN PLATELET THROMBI *in Vitro*[a]

Concn of PGE_1, μg/ml	Time at which thrombi dispersed	Time at which lysis occurred
Control (saline)	>1200	>1200
10	182.0 ± 6.7 (−85%)[b]	401.0 ± 35.0 (−67%)[b]
5	172.8 ± 7.0 (−86%)[b]	350.0 ± 4.7 (−71%)[b]
1	136.8 ± 3.9 (−87%)[b]	340.5 ± 17.4 (−72%)[b]
0.1	144.5 ± 8.5 (−88%)[b]	366.5 ± 31.0 (−69%)[b]
0.05	235.0 ± 16.5 (−80%)[b]	715.0 ± 50.2 (−40%)
0.01	453.5 ± 179.1 (−62%)	113.8 ± 54.2 (−7%)
0.005	757.0 ± 15.1 (−37%)	>1200
0.001	1078.0 ± 45.6 (−10%)	>1200

[a] PGE_1 is added 30 sec after addition of ADP; lysis time in seconds is expressed as mean ± standard error; four samples for each concentration of PGE_1. [b] Statistically significant; $p < 0.05$ by Student's t test; control value is taken as 1200 for calculating percentage reduction in experimental samples.

To investigate whether PGE_1 would affect platelet behavior *in vivo*, rats (six per group) were given a single intravenous injection of 3 mg of PGE_1/kg or saline. Table IV shows that platelet aggregation in PRRP was significantly inhibited up to 30 min following injection. Lower doses of PGE_1 showed shorter duration of activity. It was also observed that 3 mg of PGE_1/kg was too high a dose for the animals to tolerate without overt signs of discomfort. Since single injections of lower doses did not produce significant inhibition of platelet aggregation and since single injections of high doses produced undesirable acute effects, the effect of continuous infusion of a low dose of PGE_1 (0.18 mg/kg infused over a 5-min period, ten times each day) for 30 days was investigated in rats. Results in Table V show that VA, SS, and PH were significantly inhibited in the PGE_1-infused rats.

Results obtained with rats given PGE_1 orally showed that the compound did not affect platelet aggregation of animals even at massive doses of 10 mg/kg body weight.

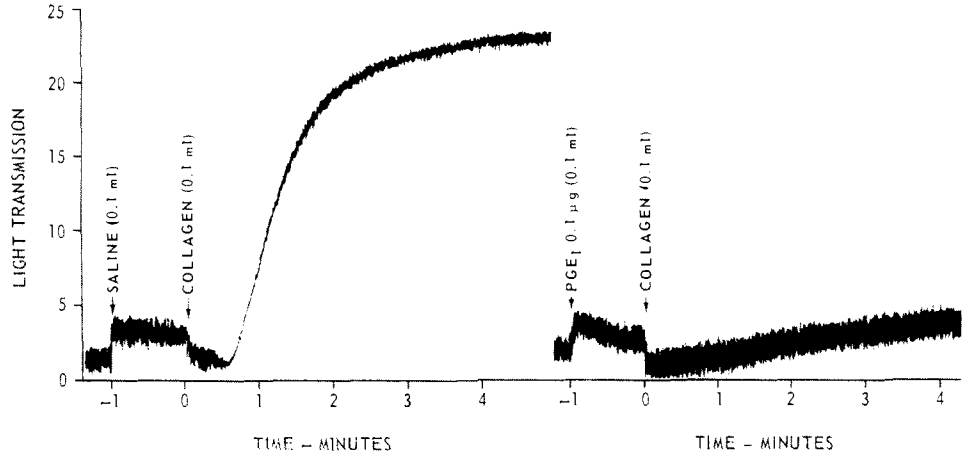

Figure 1.—Inhibition of collagen-induced aggregation-adhesion in PRHP by PGE_1.

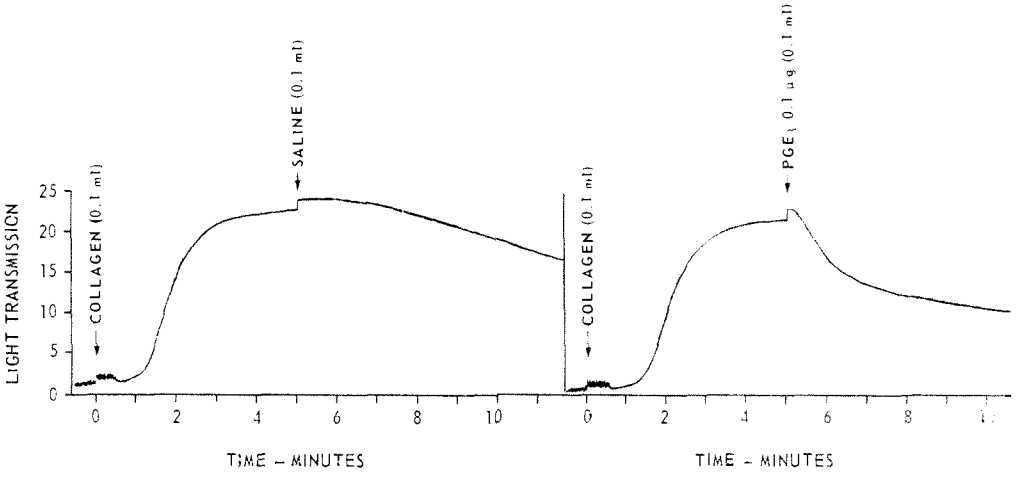

Figure 2.—Lysis of collagen-induced platelet aggregates in PRRP by PGE_1.

Discussion

It was reported by Kloeze[4] that PGE_1 inhibits ADP-induced aggregation of rat and human platelets. He also observed that PGE_2 accelerates ADP-induced aggregation of rat platelets and that the compound showed no significant effect on human platelets. Emmons and coworkers[8] have confirmed Kloeze's findings with PGE_1 and have further reported that topical applications of PGE_1 solution and intravenous injection of PGE_1 temporarily inhibited "injury-induced platelet thrombi formation" in cerebral cortical arteries of rabbits. Our investigations on the effect of prostaglandins on platelet aggregation induced by three agents (ADP, thrombin, or collagen) showed that all prostaglandins possessed some degree of inhibitory activity, though PGE_1 was by far the most potent compound in the group. Data in Tables I and II show that at least two of the prostaglandins (PGE_2 and $PGF_{1\alpha}$) are more active in PRRP than in PRHP. In addition, PGA_2 is active at 70 μg/ml against human and rat platelet aggregation (Tables I and II), whereas it is inactive against rabbit platelet aggregation even at 100 μg/ml.[5] These observations suggest that the different prostaglandins show a possible species difference in their inhibition of platelet aggregation.

PGE_2, though not as active as PGE_1, inhibited platelet aggregation of rat and human plasmas at concentrations of 12.5 and 20 μg/ml, respectively; lower concentrations of PGE_2 (3 to 0.1 μg/ml) showed a slight potentiating effect on ADP-induced rat platelet aggregation. PGE_2 did not show any significant potentiation of ADP-induced human platelet aggregation at any of the concentrations investigated (0.01 ng/ml to 100 μg/ml). These results are in partial agreement with those of Kloeze[4] and vanCreveld and Pascha.[9] The biphasic activity of PGE_2 appears to depend on the concentrations of ADP and PGE_2. Kloeze[4] attributed the inhibitory activity he observed with higher concentration of PGE_2 to possible contamination of the sample with PGE_1. However, since the PGE_2 we used was a recrystallized sample (homogenous by thin layer chromatography), the biphasic activity observed with PGE_2 in our studies appears to be a true effect of the compound.

The ability of PGE_1 to accelerate lysis of the platelet thrombi induced by ADP and its ability to partially disperse collagen-induced platelet aggregates suggest that aggregation–adhesion reactions induced by both these agents are at least partially reversible by PGE_1.

Though PGE_1 is quite stable at room temperatures and retains its inhibitory activity against platelet

(8) P. R. Emmons, J. R. Hampton, M. J. R. Harrison, A. J. Honour, and J. R. A. Mitchell, *Brit. Med. J.*, **2**, 468 (1967).

(9) S. vanCreveld and C. N. Pascha, *Nature*, **218**, 361 (1968).

TABLE IV
INHIBITION OF PLATELET AGGREGATION IN RATS GIVEN INTRAVENOUS INJECTION OF PGE_1 (3 MG/KG)[a]

Time elapsed between inject. and sample collection, min	Aggregation in recalcified plasma (thrombin-induced), time in sec[b] VA	SS	PH	TF	Collagen-induced aggregation,[b] max level
0 (control)	17.0 ± 0.5	23.1 ± 0.7	27.8 ± 0.8	198.8 ± 11.8	17.9 ± 1.0
5	25.8 ± 1.5 (+52%)[c]	159.7 ± 18.8 (+591%)[c]	183.5 ± 19.7 (+560%)[c]	208.0 ± 17.6 (+5%)	12.5 ± 1.6 (−30%)[c]
10	23.8 ± 0.9 (+40%)[c]	106.2 ± 25.7 (+360%)[c]	172.7 ± 18.7 (+521%)[c]	200.2 ± 15.5 (+1%)	10.4 ± 2.4 (−42%)[c]
15	18.8 ± 0.5 (+11%)	35.0 ± 3.3 (+52%)	94.8 ± 27.2 (+241%)[c]	235.3 ± 14.2 (+18%)	13.4 ± 0.8 (−26%)[c]
30	18.0 ± 0.9 (+6%)	27.7 ± 3.3 (+20%)	89.8 ± 38.6 (+223%)[c]	208.2 ± 9.1 (+5%)	12.6 ± 1.9 (−30%)[c]
60	17.0 ± 0.6	23.8 ± 0.8 (+3%)	29.7 ± 1.3 (+7%)	183.3 ± 7.0 (−8%)	17.6 ± 0.6 (−2%)

[a] Six rats per group. [b] Values expressed as mean ± standard error. [c] Statistically significant, $p < 0.05$ by Student's t test.

TABLE V
EFFECT OF PROLONGED PGE_1 INFUSION (30 DAYS) ON PLATELET AGGREGATION OF NORMAL RATS[a]

Animal group	VA	SS	PH	TF	Prothrombin time
Control	16.5 ± 1.7	22.5 ± 2.4	27.8 ± 2.9	305.8 ± 24.1	14.0 ± 0.1
PGE_1 infused (1.8 mg/kg/day)	20.1 ± 0.8 (+22%)[b]	27.3 ± 1.1 (+21%)[b]	37.3 ± 2.0 (+34%)[b]	334.3 ± 14.1 (+9%)	14.5 ± 0.2 (+3%)

[a] Four rats in control group and eight rats in experimental group; platelet aggregation and prothrombin time in seconds expressed as mean ± standard error. [b] Statistically significant, $p < 0.05$ by Student's t test.

aggregation even after several hours of incubation with platelet-rich plasma or whole blood,[10] the compound loses its activity within a few minutes after injection into the rat. Apparently PGE_1 is rapidly destroyed *in vivo* by tissue enzymes. The observation of Ferreira and Vane[11] that intravenously infused PGE_1 rapidly disappears from circulating blood (as determined by the smooth muscle activity and blood pressure changes), conforms with our observation that the inhibitory activity of intravenously infused PGE_1 on platelet aggregation is of very short duration. Experiments with orally administered PGE_1 showed that even very high doses did not produce any effect on platelet aggregation in animals. Recent observations of Parkinson and coworkers[12,13] suggest that PGE_1 is metabolized extensively by rat jejunum, which could possibly explain the absence of any effect on platelet aggregation in rats given oral doses of PGE_1. Since the intravenous activity was short lived and because high doses were not tolerated by the animals without undesirable effects, continuous infusion of low doses of PGE_1 was considered as a feasible method to maintain the inhibitory activity of PGE_1 against platelet aggregation. Results presented in this paper (Table V) show that platelet aggregation can be prolonged significantly in rats, by continuous infusion of well-tolerated doses of PGE_1. Mention may be made here of the recent report of Carlson and coworkers[14] who could not detect any inhibitory activity on platelet aggregation in three human volunteers given continuous PGE_1 infusion at the rate of 0.1 μg/kg per min for 30 min. Though 0.1 μg/kg per min might be too low a dose to produce significant inhibition of platelet aggregation in humans, these workers observed that higher doses produced undesirable side effects of nausea, headache, and flushing.

Though most of the prostaglandins are vasodilators, their hypotensive activity does not seem to parallel their inhibitory activity on platelet aggregation. For example, in rats PGE_1 is a potent hypotensive while $PGF_{2\alpha}$ is a pressor agent;[15] yet both inhibit platelet aggregation. Similarly, though PGE_1 and PGE_2 are comparable in their blood pressure lowering effect,[16] PGE_1 is at least 100 times more active that PGE_2 in inhibiting platelet aggregation (Tables I and II).

PGE_1 is known to be a very potent lipolysis inhibitor.[17,18] It has also been reported that lipolytic agents and elevated free fatty acid levels in blood accelerate platelet aggregation and the coagulation process.[19,20] However, the observation[21] that lipolysis inhibitors like nicotinic acid, 5-methylpyrazole-3-carboxylic acid, and 3-methylisoxazole-5-carboxylic acid did not produce any significant effect on rat platelet aggregation *in vitro* (100 μg/ml) or *in vivo* (100 mg/kg single dose in fasted rats) suggests that the antilipolytic activity of PGE_1 may not have any direct relation to its inhibitory activity on platelet aggregation. It is possible then that PGE_1 is acting on the platelet surface so that the cells become resistant to the external aggregation stimuli. Since PGE_1 can also rapidly lyse ADP-induced platelet head, it is conceivable that platelets take up PGE_1 preferentially, which alters the platelet surface charge and nullifies the ADP effect. It is important to note

(10) N. Chandra Sekhar, unpublished observations.
(11) S. H. Ferreira and J. R. Vane, *Nature*, **216**, 868 (1967).
(12) T. M. Parkinson, J. C. Schneider, Jr., J. J. Krake, and W. L. Miller, *Life Sci.*, **7**, 883 (1968).
(13) T. M. Parkinson and J. C. Schneider, Jr., *Biochim. Biophys. Acta*, **176**, 78 (1969).
(14) L. A. Carlson, E. Irion, and L. Orö, *Life Sci.* **7**, 85 (1968).
(15) D. W. DuCharme, J. R. Weeks, and R. G. Montgomery, *J. Pharmac. Exp. Ther.*, **160**, 1 (1968).
(16) S. Bergström, *Science*, **157**, 382 (1967).
(17) D. Steinberg, M. Vaughan, P. Nestel, and S. Bergström, *Biochem. Pharmacol.*, **12**, 764 (1963).
(18) S. Bergström, L. A. Carlson, L. G. Ekelund, and L. Orö, *Biochem. J.*, **92**, 42P (1964).
(19) W. E. Connor, J. C. Hoak, and E. D. Warner, *J. Clin. Invest.*, **42**, 860 (1963).
(20) J. C. Hoak, J. C. Poole, and D. S. Robinson, *Amer. J. Pathol.*, **43**, 987 (1964).
(21) N. Chandra Sekhar, unpublished observations.

that PGE_1 is a unique compound that inhibits platelet aggregation induced by ADP, thrombin, and collagen; it is also active when administered intravenously, and it lyses formed platelet thrombi. The vast difference in antithrombotic activity between PGE_1 and closely related structures shows that minor modifications in the structure of prostaglandin can greatly alter the antithrombotic properties of the compound. This observation would suggest that it should be possible to modify the prostaglandin molecule further to yield an analog with potent antithrombotic properties, but with fewer side effects than PGE_1. Even if such an analog has only a short duration of activity, as in the case of PGE_1, it might be feasible to administer the compound clinically as a continuous intravenous infusion to inhibit platelet aggregation and other thromboembolic complications.

Acknowledgment.—The author thanks Mr. Daniel J. Wynalda for expert technical assistance, Dr. James R. Weeks for helping with the infusion experiments, and Drs. John E. Pike, E. G. Daniels, and F. H. Lincoln for the prostaglandins.

The Inhibition of Viral Neuraminidase by 1-Phenoxymethyl-3,4-dihydroisoquinolines. I. Steric Effects

MICHAEL S. TUTE, KEITH W. BRAMMER, BARRY KAYE, AND REGINALD W. BROADBENT

Research Division, Pfizer Ltd., Sandwich, Kent, England

Received May 5, 1969

A series of 1-phenoxymethyl-3,4-dihydroisoquinolines were shown to be noncompetitive inhibitors of influenza virus neuraminidase. A structure–activity study revealed severe steric limitations on binding to a hypothetical receptor on the enzyme, the preferred conformation for binding being that where the phenyl and isoquinoline ring systems are approximately coplanar, and the oxygen and nitrogen atoms are cisoid. Corresponding isoquinolines and 1,2,3,4-tetrahydroisoquinolines are inactive. A novel dehydrogenation reaction has been discovered, which apparently proceeds *via* the N-oxide of the 3,4-dihydroisoquinoline.

The enzyme neuraminidase (N-acetylneuraminate glycohydrolase, EC 3.2.1.18) is present as a component of the surface protein in all strains of influenza and parainfluenza viruses, these viruses representing a large proportion of the common respiratory pathogens.[1]

Investigations[2–5] into the function of the enzyme in influenza virus replication have provided evidence that it assists in the release of virus from infected cells. Inhibition of the enzyme would be expected to slow down the rate of release, thus delaying the spread of virus within the host tissue. A search for inhibitors was therefore made as a rational approach to the chemotherapy of influenza, and this paper describes studies based on the discovery of inhibition by 1-phenoxymethyl-3,4-dihydroisoquinoline (**5**, R = H). Compounds within this series possessed considerable antiviral activity *in vitro*.[6] The compound (**5**, R = OCH_3) was subsequently tested in man and found to have a prophylactic effect against an influenza B infection.[7]

Structure–Activity Relationship.—It was soon established that simple *para* substitution in the phenyl ring to give compounds **5** (R = Cl, OCH_3) resulted in a significant but undramatic increase in enzyme-inhibitory activity. We therefore made a study of the effect of certain structural changes upon the binding of the chosen prototype (**5**, R = Cl) to a receptor on the enzyme, assuming that a comparison of levels of inhibition of the enzyme by compounds at equimolar concentrations provided a measure of their relative affinities for the receptor. It was hoped that in this way we could determine both steric and electronic limitations on binding, and eventually predict more active molecules. Chart I represents the most readily obtained related structures, and of these only the methiodide **7** was found to retain some activity. The N atom of the intermediate amide **4** and of the N-formyltetrahydroisoquinoline **2** is no longer basic, and so it was at first assumed that the nitrogen should be in a form capable of ionization, but the inactivity of the tetrahydroisoquinoline **1** (a strong base) and of the isoquinoline **8** was against this assumption, as was the fact that the inhibition of enzyme by **5** was found to be independent of pH, when this was varied from pH 5.5 to 7.5, the pK_a of **5** (R = Cl) being 6.57.[8] The inactivity of **1** and **8** is better explained on steric grounds, for an examination of molecular models[9] shows that the relative positions in space of the two aromatic nuclei, and of the N and O atoms, all possible binding sites, are different in compounds **5**, **1**, and **8**.

Compounds **10**, **11**, and **12** were prepared in order to confirm that the phenyl ring and oxygen atom were necessary for binding. All were inactive at the standard concentration of 2 m*M*, but slight inhibitory activity was found for **12** (in which CH_2 replaces O) at 5 m*M*. The function of the oxygen may be to furnish a stable constrained conformation which places the phenyl group at an aryl-binding site on the receptor.

Consideration of a model of **5** led us to the hypothesis

(1) J. E. Banatvala, *Proc. Roy. Soc. Med.*, **60**, 637 (1967).
(2) B. L. Padgett and D. L. Walker, *J. Bacteriol.*, **87**, 363 (1964).
(3) J. T. Seto and R. Rott, *Virology*, **30**, 731 (1966).
(4) R. G. Webster and W. G. Laver, *J. Immunol.*, **99**, 49 (1967).
(5) E. D. Kilbourne, W. G. Laver, J. L. Schulman, and R. G. Webster, *J. Virol.*, **2**, 281 (1968).
(6) K. W. Brammer, C. R. McDonald, and M. S. Tute, *Nature*, **219**, 515 (1968).
(7) A. S. Beare, M. L. Bynoe, and D. A. J. Tyrell, *Lancet*, i, 843 (1968).
(8) Measured by the pH method as in A. Albert and E. P. Serjeant, "Ionization Constants of Acids and Bases, Laboratory Manual," John Wiley & Sons, Inc., New York, N. Y., 1962.
(9) Courtauld space-filling models.