Table 2 demonstrates the inhibitory effect of cycloheximide on sulfamethoxypyridazine-induced stimulation of PAH accumulation in renal cortical slices from adults. The normally observed PAH accumulation is not affected by cycloheximide. A similar effect of cycloheximide on probenecid-induced stimulation was noted.

Discussion. In principle, a PAH slice to medium ratio > 1 measured in renal cortical slices is an index of the ability of the proximal tubular cells to maintain a concentration gradient. Under steady-state conditions, PAH accumulation is the result of a carrier-mediated transport into the tubular cells, a possible intracellular retention, and of the efflux from the cells back into the incubation medium. Energy is also required to keep the transported PAH inside the cellular border[8]. A passive PAH uptake cannot be stated[8,12]. The intracellular PAH portion is in a free form[13].

Enhancement of PAH accumulation in renal cortical slices from rats repeatedly administered various organic anions could prove a substrate-induced stimulation of the transport system of organic anions. Changes in glomerular filtration rate, renal blood flow, or in extrarenal factors can be excluded.

In our experiments repeated administrations of PAH, probenecid, phenol red, sulfamethoxypyridazine, and cyclopenthiazide to newborn, infant, and adult rats produced an enhancement of PAH accumulation in renal cortical slices from adult, but not from 5- and 15-day-old animals. Similar results were found for THAM accumulation following repeated THAM administrations[9]. There is a good agreement of results obtained under in vitro- and in vivo-conditions[2,14]. On the other hand, repeated administrations of saline or of the organic cation THAM to adult rats has no effect on the accumulation of the organic anion PAH. Consequently, enhancement of renal tubular PAH transport can be interpreted as substrate-induced stimulation.

After repeated administrations of PAH, probenecid, phenol red, sulfamethoxypyridazine, and cyclopenthiazide, no symptoms for nonspecific toxic effects of these drugs occured[15].

Stimulation of the organic acid transport system by penicillin, PAH, and gentamicin, respectively[16-19] was already documented. Treatment of rats with nontoxic doses of organic cationic substrates, such as N-methylnicotinamide or tetraethylammonium, did not result in stimulatory effects, whereas treatment of rats with nephrotoxic agents, such as uranyl nitrate or potassium dichromate specifically enhanced N-methylnicotinamide accumulation in renal cortical slices[20,21].

The observed enhancement of the carrier-mediated PAH transport could be produced by an increased concentration of carrier protein or by an increased turnover rate of the carrier. Consequently, it was studied whether or not the stimulation of PAH accumulation in renal cortical slices can be prevented by simultaneous administration of an inhibitor of protein synthesis[6]. Cycloheximide significantly inhibits the stimulatory effects induced by sulfamethoxypyridazine as well as probenecid. An effect on the de novo-synthesis of carrier proteins can be supposed, as already postulated[22]. Furthermore, after repeated administrations of various organic anions the apparent Michaelis constant (i.e. the affinity of PAH for the transport sites) is unchanged, whereas the maximum PAH concentration (i.e. the transport capacity or transport velocity) is increased in renal cortical slices[18,23]. Finally, the PAH efflux from the proximal tubular cells back into the incubation medium is not affected by the pretreatment of rats with PAH, cyclopenthiazide, and sulfamethoxypyridazine[23]. Further ways and means must be found to characterize the functional transport sites as well as the energy availability.

1 Acknowledgment: The authors thank Miss Karin Müller for technical assistance.

2 R. Storch and H. Bräunlich, Acta biol. med. germ. *34,* 1529 (1975).
3 H. Bräunlich and R. Storch, Acta biol. med. germ. *35,* 1411 (1976).
4 M. Stopp, Chr. Weise, S. Mewes and H. Bräunlich, Acta biol. med. germ. *35,* 787 (1976).
5 E.B. Berkhin and B.Y. Varshavsky, Proc. Acad. Sci., USSR, *220,* 1463 (1975).
6 D.G. Pegg and J.B. Hook, J. Pharmac. exp. Ther. *195,* 16 (1975).
7 M. Stopp and H. Bräunlich, Z. Versuchstierk. *16,* 131 (1974).
8 M. Stopp and H. Bräunlich, Acta biol. med. germ. *34,* 89 (1975).
9 M. Stopp and H. Bräunlich, Experientia *32,* 1182 (1976).
10 A.C. Bratton and E.K. Marshall, Jr, J. biol. Chem. *128,* 537 (1933).
11 W.H. Waugh, J. appl. Physiol. *37,* 752 (1974).
12 M. Stopp and H. Bräunlich, Agressologie *16,* 373 (1975).
13 E.C. Foulkes, Am. J. Physiol. *205,* 1019 (1963).
14 H. Bräunlich, K. Luther and S. Rudolph, Experientia *30,* 1314 (1974).
15 D.G. Pegg, K.M. McCormack and J.B. Hook, Experientia *32,* 1315 (1976).
16 G.H. Hirsch and J.B. Hook, Science *165,* 909 (1969).
17 G.H. Hirsch and J.B. Hook, J. Pharmac. exp. Ther. *171,* 103 (1970).
18 J.T. Bond, M.D. Bailie and J.B. Hook, J. Pharmac. exp. Ther. *199,* 25 (1976).
19 R. Lapkin, R. Bowman and G.J. Kaloyanides, J. Pharmac. exp. Ther. *201,* 233 (1977).
21 G.H. Hirsch, Can. J. Physiol. Pharmac. *50,* 533 (1972).
21 G.H. Hirsch and A.P. Pakuts, Toxic. appl. Pharmac. *32,* 109 (1975).
22 G.H. Hirsch and J.B. Hook, J. Pharmac. exp. Ther. *174,* 152 (1970).
23 M. Stopp and H. Bräunlich, in preparation.

Radioimmunological determination of prostaglandin D_2 synthesis in human thrombocytes[1]

H. Anhut, B.A. Peskar, W. Wachter, B. Gräbling and B.M. Peskar

Pharmakologisches Institut und Medizinische Klinik der Universität Freiburg i. Br., D-7800 Freiburg i. Br. (Federal Republic of Germany), 19 April 1978

Summary. A specific radioimmunoassay for prostaglandin D_2 was developed. Using the radioimmunoassay, prostaglandin D_2 synthesis by human thrombocytes was measured. While the cyclooxygenase inhibitor indomethacin inhibits formation of prostaglandin D_2, increased formation of prostaglandin D_2 was observed in the presence of the thromboxane synthetase inhibitor imidazole.

Prostaglandin (PG) D_2 has been considered as a biological inactivation product of the PG endoperoxide PGH_2[2] from which it can be formed by enzymatic or non-enzymatic isomerization[2-4]. Recently, however, a number of interesting pharmacological effects of PGD_2 have been described, such as inhibition of platelet aggregation[5,6], increase of

canine renal blood flow[7] and reduction of coronary flow in isolated perfused guinea-pig hearts[8,9]. For quantitative determination of endogenous PGD_2 in biological material, bioassays[10,11] and gas chromatography/mass spectrometry[4,12] have been used. We have now developed a radioimmunoassay for PGD_2. Using the radioimmunoassay we have measured PGD_2 synthesis by washed human platelets after addition of thrombin, and the effects of the cyclooxygenase inhibitor indomethacin[13] and the thromboxane (TX) synthetase inhibitor imidazole[14].

Material and methods. PGD_2 as well as the other PGs and TXB_2 were generous gifts of Dr J. Pike (Upjohn Co, Kalamazoo, USA). PGD_2 was made immunogenic by coupling to bovine serum albumin (BSA, Calbiochem, San Diego, USA) according to the method of Axen[15]. Briefly, after incubation of 1.5 mg PGD_2 with 0.7 mg N,N'-carbonyldiimidazole (Merck-Schuchardt, München, FRG) in 0.2 ml dimethylformamide at room temperature for 1 h, 4.0 mg BSA in 0.3 ml aqua dest. was added and incubation continued for 7 h. Then the preparation was dialyzed exhaustively, first against a 3:2 mixture of aqua dest./dimethylformamide, and then against aqua dest. alone. Aliquots of the immunogen were diluted with saline and emulsified with equal volumes of complete Freund's adjuvant (Difco, Detroit, USA). The emulsions were injected into the foot pads of rabbits (250 µg of antigen/animal). Booster injections with 100 µg of antigen/animal were given 1 and 3 weeks later and then in monthly intervals. Blood was taken by puncture of the ear artery 10–14 days after booster injections.

The blood was collected into 7.5% (v/v) of 77 mM sodium EDTA plus 0.1 mM indomethacin (final concentration) to inhibit synthesis of PGs and TXB_2 by thrombocytes. The antiplasma was separated from the blood cells by centrifugation (1000 × g, 15 min).

The labelled ligand for the PGD_2 radioimmunoassay was prepared by a method similar to that described for the synthesis of unlabelled PGD_2 by Nugteren and Hazelhof[2]. Lyophilized sheep seminal vesicle microsomes (2 mg) as enzyme source were incubated with 0.5 mCi of 5,6,8,9,11,12,14,15-^{3}H-arachidonic acid (New England Nuclear Co, Dreieichenhain, FRG, sp. act. 98.5 Ci/mmole) as substrate in the presence of 10 µg hydrochinon in a total volume of 0.22 ml at 37 °C for 6 h. After acidification to pH 3.2 with 0.01 N HCl, the various reaction products and remaining substrate were extracted into 2 × 5 ml diethyl

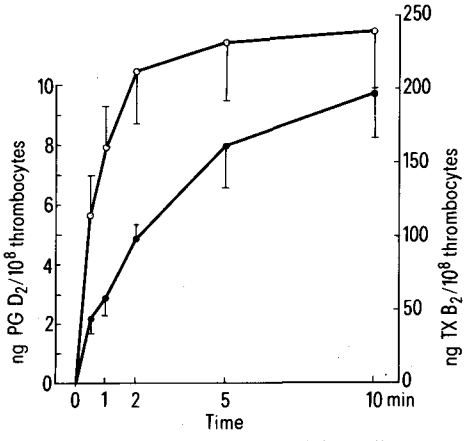

Fig. 1. Release of TXB_2 (●——●, right ordinate) and PGD_2 (○——○, left ordinate) from washed human thrombocytes at 37 °C after addition of thrombin. The results represent the means±SEM of 5 experiments.

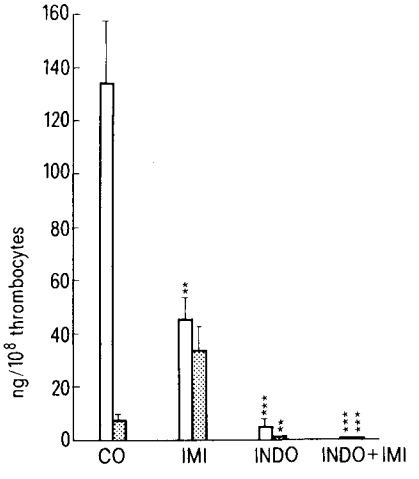

Fig. 2. Effect of indomethacin (4 µM) and imidazole (4 mM) on TXB_2 (open bars) and PGD_2 (hatched bars) release from washed human thrombocytes after addition of thrombin. The results represent the means ± SEM of 5 experiments. *$p<0.05$; **$p<0.01$; ***$p<0.001$. CO, Controls; IMI, imidazole; INDO, indomethacin.

Specificity of the radioimmunoassay for PGD_2

	50% displacement of bound label (ng)	Relative cross-reaction (%)
PGD_2	3.5	100.0
PGD_1	7.5	46.7
PGE_2	200	1.8
PGE_1	240	1.5
$PGF_{2\alpha}$	350	1.0
$PGF_{1\alpha}$	350	1.0
6-keto-$PGF_{1\alpha}$	475	0.7
PGA_2	> 500	< 0.7
PGA_1	> 500	< 0.7
TXB_2	> 500	< 0.7
15-keto-PGE_2	> 500	< 0.7
15-keto-$PGF_{2\alpha}$	> 500	< 0.7
15-keto-13,14-dihydro-PGE_2	> 500	< 0.7
15-keto-13,14-dihydro-$PGF_{2\alpha}$	> 500	< 0.7

ether and then separated by TLC (solvent system diethyl ether-methanol-acetic acid 90:1:2). The labelled material co-chromatographing with authentic PGD_2 (yield about 1%) was eluted from the plate with methanol and used as tracer in the radioimmunoassay. Its sp. act. was not determined. Radioimmunoassays were performed as described by van Orden and Farley[16], using polyethylene glycol for separation of free and antibody-bound fractions of antigen.

Washed human thrombocytes were prepared by the method of Hamberg et al.[17]. Samples (0.2 ml) of the platelet suspension (platelet count 400,000–600,000/µl) were pre-incubated at 37 °C for 2 min and then aggregation was induced by the addition of 1 U of thrombin. The reaction was stopped after various time intervals by the addition of 2.0 ml ethanol. After centrifugation, aliquots of the supernatant were evaporated and the residues taken up in phosphate-buffered saline containing 1 mg/ml gelatine (GPBS). PGD_2 and TXB_2 in these samples were determined by radioimmunoassay. The details of the radioimmunoassay for TXB_2 have been described previously[18]. Indomethacin (Sharpe and Dohme, München, FRG) and imidazole (Merck, Darmstadt, FRG) were dissolved in 0.1 M sodium phosphate buffer, pH 7.4 and diluted in Krebs-Henseleit solution[19] not containing calcium. These enzyme inhibitors (final indomethazin concentration 4 µM, final imidazole concentration 4 mM) were added before pre-incubation in a volume of 50 µl. Appropriate controls received the same volume of solvent. In these experiments with inhibitors, incubation time was 90 sec, since under the conditions used, synthesis of PGD_2 and TXB_2 was linear for up to about 2 min in control incubates (figure 1).

Results. All rabbits immunized with the PGD_2-BSA conjugate produced antibodies against PGD_2. An antiplasma obtained about 7 months after 1st immunization could bind 35% of the added label (10 nCi) at a final dilution of 1:280. The specificity of the radioimmunoassay for PGD_2 is shown in the table. The best inhibitor of binding of label to the antiplasma is the homologous antigen PGD_2, 3.5 ng inhibiting 50%. The sensitivity of the assay allows the detection of 200 pg of PGD_2. The relative standard deviation for determination of 1–10 ng PGD_2 is ±6.0%. Of all the other compounds tested, only PGD_1 cross-reacts significantly in the radioimmunoassay (table).

The time-dependent synthesis of immunoreactive PGD_2 and TXB_2 by washed human thrombocytes after addition of thrombin is shown in figure 1. Neither TXB_2 nor PGD_2 were detected in the incubates before addition of thrombin. After addition of thrombin, the thrombocytes synthesize large amounts of TXB_2 and much smaller amounts of PGD_2. In order to validate the radioimmunoassay for PGD_2 in these experiments, it was shown that addition of various amounts of PGD_2 (1–10 ng) to the thrombocyte suspensions produced the expected increments in the radioimmunoassay.

While indomethacin (4 µM) strongly inhibited TXB_2 as well as PGD_2 synthesis in response to thrombin, the TX-synthetase inhibitor imidazole (4 mM) reduced TXB_2 synthesis significantly and simultaneously increased synthesis of PGD_2 (figure 2). When indomethacin was added together with imidazole, again inhibition of both TXB_2 and PGD_2 synthesis was observed (figure 2).

Discussion. The production of specific antibodies against PGD_2 has so far not been reported. The specificity of the radioimmunoassay for PGD_2 shows that the antibodies recognize the PGD ring structure as immunodominant part of the hapten molecule, while the number of double bonds in the side chains is less important for binding. Thus PGD_1 is a relatively good inhibitor in the radioimmunoassay, but PGs of the E and F series are only negligibly bound to the antibodies. Our radioimmunological data on PGD_2 formation by thrombocytes are in agreement with the results of other authors[10,12]. The complete inhibition by indomethacin of PGD_2 and TXB_2 synthesis as measured by radioimmunoassay shows that the results are not invalidated by material in the incubates which could interfere non-specifically with the antigen-antibody reactions.

Oelz et al.[10] using bioassay as well as mass spectrometry described the synthesis of PGD_2 by human thrombocytes in response to a number of aggregating agents including thrombin. They concluded from their results that much of the total PGD_2 found may be formed enzymatically in thrombocytes. They also discussed the possible action of the PGD_2 synthesized as a feed-back inhibitor of platelet aggregation in vitro.

While the effects of cyclooxygenase inhibitors like indomethacin on platelet function have been ascribed to blockage of the formation of PG endoperoxides[17], the correlation of the antiaggregatory effect of imidazole[20] with inhibition of the enzyme TX-synthetase is controversial[21–23]. In view of the low concentrations of PGD_2 necessary to inhibit platelet aggregation[5,6,10], it seems possible that the increased formation of PGD_2 by thrombocytes in the presence of imidazole could contribute to the antiaggregatory effect of this compound under certain experimental conditions[24].

1 This work was supported by the Deutsche Forschungsgemeinschaft.

2 D.H. Nugteren and E. Hazelhof, Biochim. biophys. Acta *326*, 448 (1973).
3 M. Hamberg and B. Samuelsson, Proc. natl Acad. Sci. USA *70*, 899 (1973).
4 M.S. Abdel-Halim, M. Hamberg, B. Sjöquist and S. Änggard, Prostaglandins *14*, 633 (1977).
5 J.B. Smith, M.J. Silver, C.M. Ingerman and J.J. Kocsis, Thromb. Res. *5*, 291 (1974).
6 E.E. Nishizawa, W.L. Miller, R.R. Gorman, G.L. Bundy, J. Svensson and M. Hamberg, Prostaglandins *9*, 109 (1975).
7 P.M. Bolger, G.M. Eisner, P.T. Shea, P.W. Ramwell and L.M. Slotkoff, Nature *267*, 628 (1977).
8 K. Schrör, Naunyn-Schmiedebergs Arch. Pharmac. *302*, 61 (1978).
9 H. Anhut, W. Bernauer and B.A. Peskar, Prostaglandins *15*, 889 (1978).
10 O. Oelz, R. Oelz, H.R. Knapp, B.J. Sweetman and J.A. Oates, Prostaglandins *13*, 225 (1977).
11 S. Moncada, K.G. Mugridge and B.J.R. Whittle, Br. J. Pharmac. *61*, 451 P (1977).
12 M. Ali, A.L. Cerskus, J. Zamecnik and J.W.D. McDonald, Thromb. Res. *11*, 485 (1977).
13 J.R. Vane, Nature New Biol. *231*, 232 (1971).
14 S. Moncada, S. Bunting, J.R. Vane, K. Mullane, P. Thorogood, A. Raz and P. Needleman, Prostaglandins *13*, 611 (1977).
15 U. Axen, Prostaglandins *5*, 45 (1974).
16 D.E. van Orden and D.B. Farley, Prostaglandins *4*, 215 (1973).
17 M. Hamberg, J. Svensson, T. Wakabayashi and B. Samuelsson, Proc. natl Acad. Sci. USA *71*, 345 (1974).
18 H. Anhut, W. Bernauer and B.A. Peskar, Eur. J. Pharmac. *44*, 85 (1977).
19 H.A. Krebs and K. Henseleit, Hoppe-Seylers Z. Physiol. Chem. *210*, 33 (1932).
20 J.W. Davis and P.E. Phillips, Blood *38*, 417 (1971).
21 P. Needleman, B. Bryan, A. Wyche, S.D. Bronson, K. Eakins, J.A. Ferrendelli and Minkes, Prostaglandins *14*, 897 (1977).
22 F.A. Fitzpatrick and R.R. Gorman, Prostaglandins *14*, 881 (1977).
23 R.R. Gorman, G.L. Bundy, D.C. Peterson, F.F. Sun, O.V. Miller and F.A. Fitzpatrick, Proc. natl Acad. Sci. USA *74*, 4007 (1977).
24 F.A. Fitzpatrick and R. Gorman, Biochim. biophys. Acta *539*, 162 (1978).