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Pyridoxal 5′-phosphate—A new physiological inhibitor of blood coagulation and platelet function

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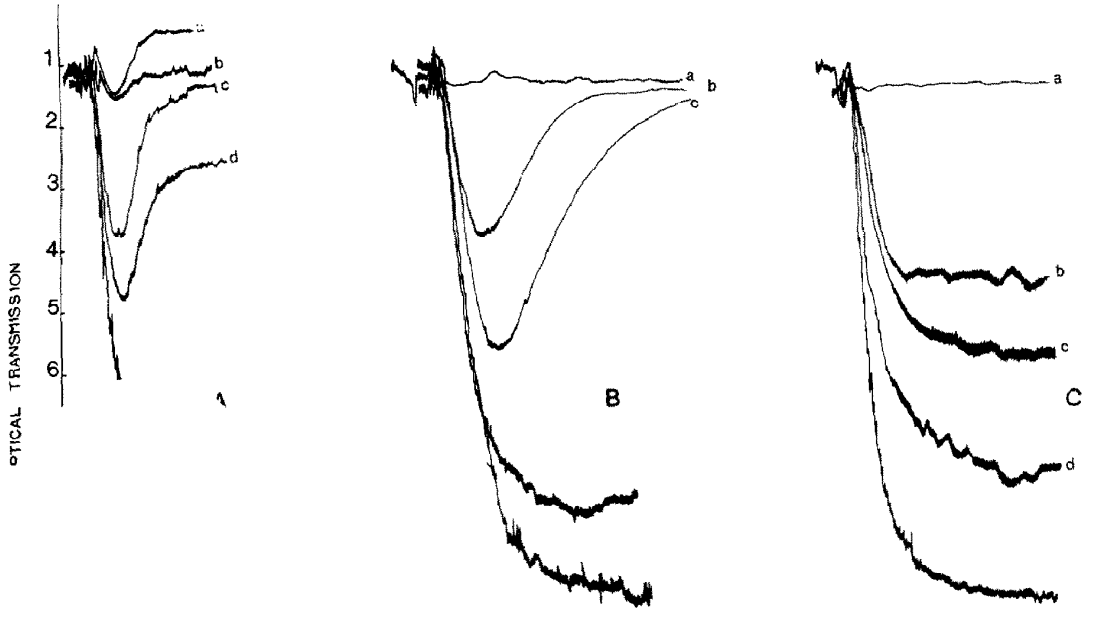
Pyridoxal 5′-phosphate (PALP) is required for many enzymatically catalyzed reactions of amino acid metabolism, glycogen catabolism and porphyrin synthesis, in which it participates through formation of a Schiff base [1]. In addition to its normal function as a cofactor for enzymes, such as transaminases and decarboxylases, PALP, with its reactive aldehyde group, has been found to be an ideal reagent for chemical modification of enzymes and proteins as a means of identifying and studying the functional groups [2–16]. In view of these findings, it is reasonable to suggest that PALP might interact with platelets and thereby modify platelet function. To investigate this possibility, we studied the effect of PALP on the aggregation of platelets by ADP, thrombin and collagen, and compared it with that of compounds closely related to PALP, such as pyridoxamine 5′-phosphate (PAMP) and pyridoxine (PA).

Materials. Bovine collagen was obtained from Worthington Biochemicals, Freehold, NJ. Bovine thrombin was from Parke Davis & Co., Detroit, MI, and adenosine diphosphate (ADP) and pyridoxal 5′-phosphate from Sigma Chemical Co., London, U.K. Pyridoxamine 5′-phosphate and pyridoxine were from Nutritional Biochemicals Inc., Cleveland, OH. 5′-Hydroxytryptamine [2^{14}C] (57 mCi/m-mole) was purchased from Amersham/Searle Corp., Arlington Heights, IL.

Methods. Venous blood, from healthy volunteers who had not taken any medication during the 2 weeks preceding blood collection, was drawn into 0.1 vol. of 3.8% sodium citrate and centrifuged at 225 *g* for 10 min to yield platelet-rich plasma (PRP). Platelet release reaction was measured by monitoring the release of [^{14}C]5-HT from platelets prelabelled with this amine [17]. The PRP (40 ml) was incubated with 1 μM [^{14}C]5-HT for 30 min at 37°. Aliquots of 0.5 ml PRP were used to study the release reaction under different test conditions.

Platelet aggregation studies were performed according to a previously described procedure [17]. The sample of PRP (0.5 ml), in a final volume of 0.5 ml, was placed in a siliconized cuvette containing a stirring bar and test reagent (PA, PAMP and PALP dissolved in phosphate buffer, pH 7.5) of a desired concentration. The cuvette was placed into the aggregometer, and ADP solution that gives a final concentration of 1 μM was added to initiate the aggregation response. In other experiments, collagen (50 μg) or thrombin (0.1 to 0.2 NIH units) was added to PRP to induce platelet aggregation response. The platelet aggregation of samples containing test reagents was compared to that observed in appropriate controls substituted with phosphate buffer, pH 7.5.

The clotting time of platelet-poor plasma (PPP) was determined with a 0.3-ml sample at 37° containing 0.1 ml PPP,



yridoxamine 5′-phosphate (PAMP) of platelet aggregation induced
g collagen. Aggregation is indicated by an increase in optical
(PRP) at 37°. The PRP (0.5 ml) was incubated with varying
ddition of aggregation agents. Shown in the figure are the
(b) 1.5 mM PALP, (c) 0.8 mM PALP and (d) 1.8 mM
) 1.6 mM PALP, (b) 9 mM PAMP and (c) 0.8 mM
M PALP, (b) 12 mM PAMP, (c) 1.5 mM PALP

0.1 ml saline, pH 7.4, and 0.1 ml thrombin (6 units/ml in 0.85% saline containing 20 mM $CaCl_2$). The clotting was initiated by the addition of thrombin to the incubation mixture and the tests were performed in triplicate by using a fibrometer coagulation timer (BBL Dickinson & Co. Cockysville, MO).

Platelet aggregation induced by ADP, thrombin and collagen in control and PALP- or PAMP-treated platelet suspension is shown in Fig. 1, panels A, B and C. The inhibitory effect of these agents increased with the concentration of the inhibitor and the preincubation time. At a concentration $\geqslant$ 0.8 mM, PALP completely inhibited the second wave of aggregation by ADP and thrombin (Fig. 1, panels A and B). A similar inhibitory effect on collagen-induced platelet aggregation was also observed, when PALP was present at a concentration of $\geqslant$ 1.5 mM (Fig. 1, panel C). When compared to PAMP, pyridoxal 5'-phosphate was found to be about ten times more effective in abolishing the secondary aggregation induced by thrombin and collagen. However, PALP was only five times more effective than PAMP in complete inhibition of the second wave of aggregation by ADP. The primary aggregation by ADP, thrombin and collagen was also abolished when PALP was present in PRP at a concentration higher than 2 mM (Fig. 1). Complete inhibition of the second wave of aggregation indicated that PALP and PAMP were able to inhibit the release reaction of the platelets. To examine this effect in greater detail, platelets prelabeled with [^{14}C]5-HT were incubated with varying concentrations of PALP and PAMP for 30 min at 37° and tested for the release reaction with ADP, thrombin and collagen (Table 1). A marked depression in the release of [^{14}C]5-HT from platelet preincubation with PALP and PAMP was observed. The inhibition of [^{14}C]5-HT release from platelets showed a dependence on the concentration of PALP and PAMP. Complete inhibition of [^{14}C]5-HT release was obtained at PALP levels of 0.8 to 2.0 mM, depending on the platelets of the individual donor and the type of aggregating agent (Table 1). A similar inhibitory effect on release was observed at PAMP levels of 1.8 to 10 mM. A comparison of the action of PALP and pyridoxine (PA) on the aggregation of platelets by ADP, thrombin and collagen is shown in Fig. 2. Pyridoxine inhibited the secondary aggregation of platelets by these agents, but only when present at a concentration above 10 mM. Once again, the inhibitory effect of PA showed a dependence on the concentration of the inhibitor. It is shown in Fig. 2, that PALP also suppressed the thrombin-induced coagulation of PRP. At a concentration of $\geqslant$ 0.8 mM, PALP significantly reduced the coagulation of PRP. A similar inhibitory effect was observed with pyridoxine, but only at concentrations of $\geqslant$ 10 mM. It seems reasonable to suggest that PALP ($\geqslant$ 0.8 mM) can inhibit not only platelet aggregation, but also coagulation of blood. As expected, PALP exerted a concentration-dependent prolongation on the coagulation time of human plasma (Table 2). This effect was also dependent on the preincubation time. The coenzyme also inhibited the clotting of whole blood in a concentration-dependent fashion (Fig. 3). The clotting time of whole blood was increased from 7 to 120 min when the PALP content of the blood was increased from 0 to 300 μg. A similar effect of PALP was not observed when tested with phosphate buffer.

Table 2. Effects of PALP on coagulation time of human platelet-poor plasma*

PALP concn (mM)	Clotting time (sec)
0	20 * 1
0.5	36
1.0	140
2.0	> 200

* Clotting time was determined with a 0.3-ml sample at 37° containing 0.1 ml PPP, 0.1 ml saline, pH 7.4, and 0.1 ml of bovine thrombin (6 units/ml in 0.85% saline containing 20 mM $CaCl_2$). The values are the average of three determinations.

We have shown that *in vitro* aggregation and [^{14}C]5-HT release by ADP, thrombin and collagen are inhibited by pyridoxine, pyridoxamine 5'-phosphate and pyridoxal 5'-phosphate, the last being the most effective. Since platelet

Table 1. Effect of pyridoxal 5'-phosphate and pyridoxamine 5'-phosphate on the release of [^{14}C]5-HT from platelets*

Aggregating agent	PALP concn (mM)	[^{14}C]5-HT release (% of control)	PAMP concn (mM)	[^{14}C]5-HT release (% of control)
ADP (1 μM)	0	39 + 5.0	0	40 + 6.0
	0.5	20	0.9	22
	0.8	5	1.8	5
	1.5	0	3.0	0
Thrombin (0.2 units)	0	85 + 6.0	0	83 + 3.0
	0.5	36	0.9	83
	0.8	4	2.0	80
	1.5	0	10.0	0
Collagen (50 μg)	0	71 + 8.0	0	64 + 4.0
	0.5	50	0.9	32
	1.5	30	2.0	30
	2.0	10	4.0	24
	2.5	0	7.0	0

* Platelets prelabeled with [^{14}C]5-HT were treated with PALP and PAMP of concentrations and subsequently challenged with aggregating agents presented are the means of four experiments for inhibitor-containing sions, and the mean of five determinations. + S.E. for inhibitors.

function depends on metabolically available ATP in the cell [18, 19], a reduction in the level of ATP can lead to a corresponding reduction in the amount of material released from platelets. This, together with other evidence that PALP inhibits the activity of malate and glutamic dehydrogenases [3–8] and also several glycolytic and pentose shunt enzymes [20], raises the possibility that PALP might have altered platelet function by blocking uptake of glucose and its metabolism by platelets, presumably by its action on platelet hexokinase [2] and other enzymes of the glycolytic pathway. Such a phenomenon can reduce the sensitivity of platelets to different aggregating agents. In fact, it has been shown that rabbit platelets resuspended in a solution free of glucose lose their sensitivity to aggregation by different stimuli [21].

The effect of PALP on platelet aggregation could be due to its binding to platelet membrane protein(s) or its action on platelet enzymes responsible for glucose metabolism and oxidative phosphorylation, or both. It is probable that PALP formed a Schiff base or an azolidine ring [3] with the lysine moiety or platelet membrane protein(s). Such a process might alter platelet sensitivity to different aggregating agents. Our observation that PALP is a more potent inhibitor of platelet aggregation and release reaction than PA and PAMP strongly indicates the importance of the reactive aldehyde and phosphate groups of the PALP molecule. The aldehyde and phosphate groups of PALP appear to confer greater specificity on the molecule for its interaction with the platelets. Recent investigations have indicated the important role of cyclic nucleotides in the functioning of platelets [22–24]. It would be of great interest to learn how PALP affects the level of cyclic nucleotides in the platelets.

If PALP is indeed the inhibitor of platelet aggregation and release reaction, then platelet function *in vivo* should depend on factors that regulate the level of this vitamin B_6 compound

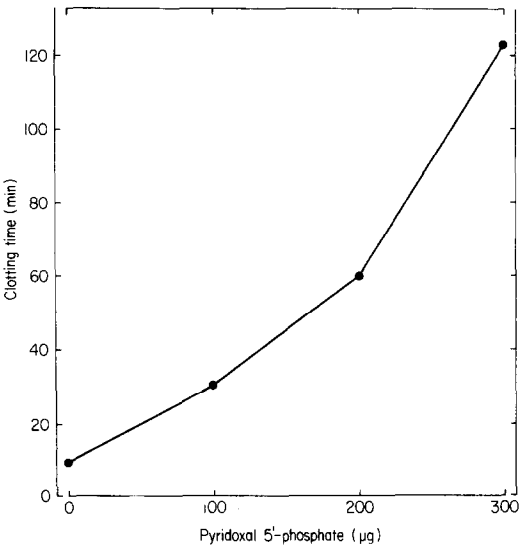


Fig. 3. Effect of PALP on the clotting time of human blood. Human blood (1 ml) was collected in a glass tube containing varying amounts of PALP at pH 7.5 and allowed to stand at 27°. The clotting of blood was inhibited by PALP in a concentration-dependent manner.

in human blood. The *in vivo* and *in vitro* studies with human blood indicated that PA is rapidly taken up and metabolized by erythrocytes whereas PALP is not [25, 26]. In platelets, the metabolism of pyridoxine was found to be restricted to a

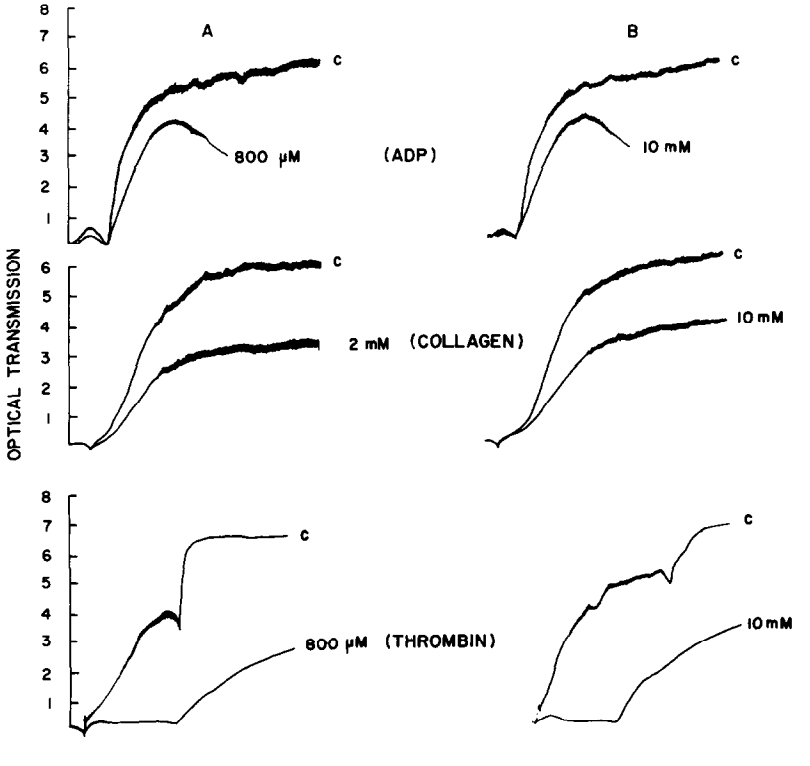


Fig. 2. Inhibition by pyridoxal 5'-phosphate (PALP) and pyridoxine (PA) of platelet aggregation induced by ADP (1 μM), thrombin (0.2 units) and collagen (50 μg). The PRP (0.5 ml) was incubated with PALP or PA for 30 min at 37° before the addition of aggregating agents. The effects of PALP (A) and PA (B) of specified concentrations on the aggregation of platelets by ADP, thrombin and collagen are shown.

reaction that converts pyridoxine to pyridoxine phosphate [27]. Previous studies demonstrated that no significant levels above > 20 ng/ml of plasma PA could be detected at any stage of incubation of PA with human blood [25]. These factors suggest that PALP is not metabolized by blood as efficiently as PA and, therefore, can remain intact in plasma for a prolonged period during circulation. If so, one can expect PALP to exert a stronger *in vivo* effect on platelet function than pyridoxine. Recently, we reported that intravenous administration of PALP to human volunteers (100 or 200 mg) markedly diminished the *ex vivo* aggregation response of platelets [28,*]. PALP also reduced the *ex vivo* coagulation of PRP by thrombin. This inhibitory effect of PALP showed a dependence on the *in vivo* incubation time and disappeared at 4 hr after the administration of coenzyme.* Since PALP is a strong inhibitor of platelet aggregation as well as coagulation of plasma both *in vitro* and *in vivo*, it may play an important role in the control of platelet sensitivity to different aggregating agents and also the coagulation of plasma.

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* K. Subbarao, H. de Haas and V. V. Kakker, manuscript submitted for publication.

† Address reprint requests to: Dr. K. Subbarao, Hematology Division, St. Jude Children's Research Hospital, Memphis, TN, U.S.A.

Specialized Center for Thrombosis Research, Department of Medicine, Temple University Medical School, Philadelphia, PA, U.S.A. — KUCHIBHOTLA SUBBARAO†

Department of Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA, U.S.A. — JAYA KUCHIBHOTLA

Thrombosis Research Unit, Kings College Hospital Medical School, London, U.K. — VIJAY V. KAKKAR