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PHOSPHORYLATION OF PYRIDOXINE BY HUMAN BLOOD PLATELETS—EFFECTS OF STRUCTURE ANALOGS AND METABOLIC INHIBITORS*

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Abstract—When incubated with ^{3}H-pyridoxine (3·0 μM) for 30 min, washed human platelets accumulated radioactivity with a gradient ([I]/[O]) of approximately 8. Such accumulation was due to the formation ^{3}H-pyridoxine phosphate which does not readily diffuse from the cell. Phosphorylation was saturable and hindered by iodoacetic acid, NaF, NaCN, DNP and congeners of pyridoxine. The gradient for unchanged pyridoxine never exceeded 1·0. Of the total platelet radioactivity, 90 per cent migrated with authentic pyridoxine phosphate and 7 per cent with pyridoxine on thin layers of silicic acid. At an initial pyridoxine concentration, in the medium of 3·0 μM, 50 per cent inhibition of the radioactivity gradient occurred with pyridoxal, 0·19 μM; pyridoxamine, 0·40 μM; 4′-deoxypyridoxine, 2·1 μM; pyridoxic acid, 500 μM; and isonicotinic acid hydrazide, 750 μM. These data suggest that pyridoxal phosphokinase has a greater affinity for pyridoxal and pyridoxamine than for pyridoxine. No pyridoxine or pyridoxine phosphate oxidase was found in the platelet and the metabolism, therein, of pyridoxine was restricted to phosphorylation.

THE HUMAN blood platelet was previously shown to synthesize nicotinamide adenine dinucleotides from nicotinic acid[1] and to accumulate folic acid.[2] The presence of aminotransferases in this cell[3] suggests it may also phosphorylate B_6 vitamers to the cofactor for these enzymes. Furthermore, the administration of pyridoxine, in large doses, to homocytinurics corrects their disordered platelet aggregation.[4] In the present study, pyridoxine was chosen to characterize the phosphorylation of vitamin B_6 by human platelets due to its stability over that of pyridoxal and pyridoxamine and its availability as a radioactive compound.

EXPERIMENTAL

The following compounds were obtained from commercial sources: pyridoxine (PN), pyridoxine phosphate (PNP), pyridoxal (PL), pyridoxal phosphate (PLP), pyridoxamine (PM), pyridoxamine phosphate (PMP), pyridoxic acid, 4′-deoxypyridoxine (4-DOP) and isonicotinic acid hydrazide (INH) from Mann Research Laboratories; isonicotinic acid and isonicotinamide from K & K Laboratories; 3-hydroxypyridine, 4-pyridinecarboxaldehyde, 3-hydroxymethylpyridine, benzaldehyde,

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benzyl alcohol, benzylamine, 3-hydroxybenzyl alcohol, 4-methylbenzyl alcohol, 3-methylbenzylamine, and pyridine from Aldrich Chemical Co. Generally labeled ^{3}H-pyridoxine (^{3}H-PN), sp. act., 950 mc/m-mole, purchased from Amersham-Searle, was found greater than 96 per cent pure on thin layers of Silica gel.[5,6]

Approximately 30 mg wet wt. of washed human platelets was suspended in 0·8 ml Ca^{2+}- and Mg^{2+}- free Krebs–Ringer bicarbonate buffer. ^{3}H-PN (0·1 ml) and 0·1 ml of solution of inhibitor or buffer were added to each suspension. Incubation was then carried out in a Dubnoff metabolic shaker under O_2–CO_2 (95:5, v/v) at 37°. After incubation, the platelets were sedimented at 22,000 *g* for 5 min, the supernatants decanted and the tubes swabbed with a cotton-tipped applicator. The samples were weighed and then lysed in 1 ml of distilled water. Aliquots (0·5 ml) of each supernatant and lysate were added to 10 ml of scintillation medium and the radioactivity was determined as previously described.[1] Counting efficiency determined by the channels ratio method was 20–25 per cent. The distribution ratio of radioactivity ([I]/[O]) was expressed as the ratio of disintegrations per minute per milliliter of intracellular platelet water to disintegrations per minute per milliliter of incubation medium. Total water was 76 and extracellular water 27 per cent of the wet wt. of the platelet pellet.[1] The concentration of radioactivity in the intraplatelet water was corrected for that trapped within the extracellular space of the platelet pellet according to the formulation of Helmreich and Kipnis.[5]

In some experiments "carrier" PN, PNP, PL, PLP, PM and PMP were immediately added to the platelet lysate and medium after incubation. After application of 100-μl aliquots onto thin layers of silicic acid, the compounds were separated,[6,7] visualized by the fluorescence quenching under ultraviolet light, scraped from the plates, suspended in gelled scintillation medium,[8] and the radioactivity was determined. The instability of PNP in platelet lysate dictated immediate chromatographic separation. Phosphates of the B_6 vitamers were also located on the chromatograms by sequential spraying with solutions of ammonium molybdate and stannous chloride.[9]

RESULTS

When incubated with ^{3}H-PN, human platelets accumulated radioactivity. At an initial concentration of 3·0 μM PN, the distribution ratio ([I]/[O]) increased with time up to 30 min and reached a maximum of approximately 10 (range 6–12) within 60 min (Fig. 1). Of the total intraplatelet radioactivity at 30 min, 90 per cent migrated with PNP and 7 per cent with PN on thin layers of silicic acid. Increasing the initial concentration of PN diminished the ratio so that saturation was approached at 60 μM where the gradient was 2 (Fig. 2).

As early as 5 min after incubation with ^{3}H-PN (3·0 μM), the distribution ratio of free PN reached and then remained 1·0 (Fig. 3). In contrast, the intraplatelet concentration of PNP increased over 1 hr; however, as the intracellular concentration approached 26 μM, phosphorylation virtually ceased. Such was the case regardless of the PN concentration in the medium.

Various metabolic inhibitors including F^-, CN^-, iodoacetic acid, *p*-chloromercuribenzoate (PCMB), and 2,4-dinitrophenol (DNP) suppressed phosphorylation of PN, and, hence, accumulation of radioactivity (Table 1). The same was true upon the addition of various PN congeners prior to incubation (Table 2). At an initial PN

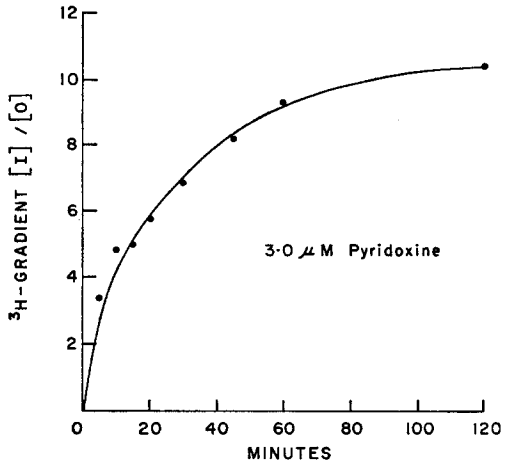

FIG. 1. Rate of accumulation of radioactivity with time by the human platelet when incubated with ^{3}H-pyridoxine (3·0 μM). Results of a representative experiment are shown.

concentration in the medium of 3·0 μM, 50 per cent inhibition of the radioactivity gradient occurred with PL, 0·19 μM; PM, 0·40 μM; 4-DOP, 2·1 μM; pyridoxic acid, 500 μM and INH, 750 μM.

The following PN congeners were inactive as inhibitors at a concentration of 1·0 mM in the presence of 3·0 μM of PN: benzyl alcohol, pyridine, 3-hydroxymethylpyridine, 3-hydroxypyridine, 4-pyridinecarboxaldehyde, 3-hydroxybenzyl alcohol, benzylamine, benzaldehyde, 4-methylbenzyl alcohol, 3-methylbenzylamine, isonicotinic acid and isonicotinamide.

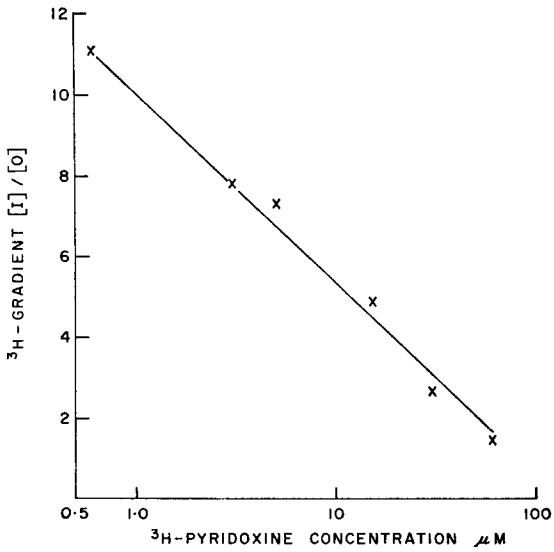

FIG. 2. Distribution, [I]/[O], of radioactivity in the human platelet when incubated with various concentrations of ^{3}H-pyridoxine for 30 min. Results of a representative experiment are shown.

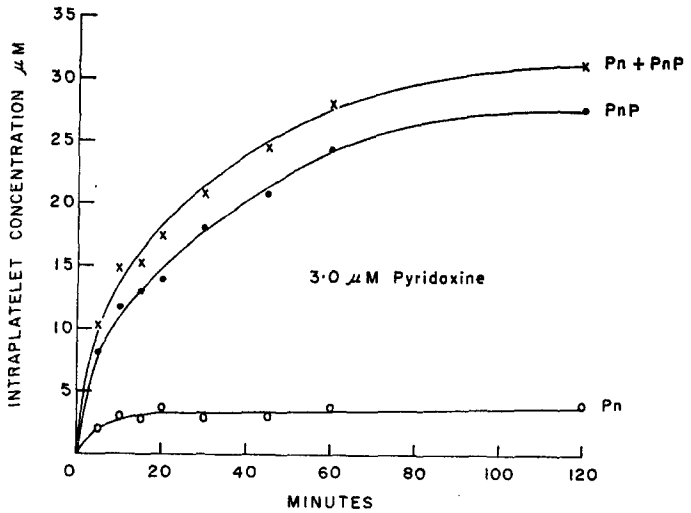

FIG. 3. Rate of accumulation of pyridoxine and pyridoxine phosphate with time in the human platelet when incubated with ^{3}H-pyridoxine (3·0 μM). Results of representative experiments are shown.

TABLE 1. INHIBITION OF RADIOACTIVITY GRADIENT, [I]/[O], AND OF PHOSPHORYLATION OF ^{3}H-PYRIDOXINE (3·0 μM) IN THE HUMAN PLATELET BY VARIOUS METABOLIC INHIBITORS (1 mM)*

Inhibitor	[I]/[O] ^{3}H gradient	Inhibition of gradient (%)	Intraplatelet concn pyridoxine phosphate†
Control	10·4	0	26
NaF	8·8	14	22
NaCN	5·5	47	14
Iodoacetic acid	5·8	44	15
PCMB	8·6	17	21
DNP	5·4	47	12

* Platelets were incubated 30 min in medium described in text. Results of representative experiments are shown.

† Micromoles per litre of intraplatelet water.

DISCUSSION

B_6 uptake by the human erythrocyte[10,11] involved mainly accumulation of the free vitamers and, to a lesser extent, phosphorylation. The present study demonstrates that the human platelet also accumulates radioactivity when incubated with ^{3}H-PN. Such accumulation is not due to PN itself, whose concentration in cellular water never differed significantly from that in the medium, but rather to the formation of PNP which does not readily diffuse from the cell. Hence, under all conditions in this study, the radioactivity gradient was due to phosphorylation of PN and, furthermore, the metabolism of PN was restricted to this reaction.

TABLE 2. CONCENTRATIONS OF VARIOUS CONGENERS OF PYRIDOXINE WHICH INHIBIT BY 50 PER CENT THE RADIOACTIVITY GRADIENT ([I]/[O]) IN THE HUMAN PLATELET WHEN INCUBATED WITH ^{3}H-PYRIDOXINE (3·0 μM) FOR 30 MIN*

Compound	Concn for 50% inhibition of gradient [I]/[O] (M)
Pyridoxal	$1{\cdot}9 \times 10^{-7}$
Pyridoxamine	$4{\cdot}0 \times 10^{-7}$
Deoxypyridoxine	$2{\cdot}1 \times 10^{-6}$
Pyridoxic acid	$5{\cdot}0 \times 10^{-4}$
Isonicotinic acid hydrazide	$7{\cdot}5 \times 10^{-4}$

* Results of representative experiments are shown.

A number of compounds inhibited phosphorylation and, therefore, the radioactivity gradient. Since phosphorylation of PN is energy dependent, it was expected and confirmed, in the platelet, that incorporation into PNP was hindered by such metabolic inhibitors as PCMB, CN^-, iodoacetate, DNP, and F^-. 4-DOP, previously shown to compete with PN for PL phosphokinase,[12–15] was also a potent inhibitor of the corresponding platelet enzyme. Although less effective, the same was true for isonicotinic acid hydrazide.[16,17] Other congeners of PN, lacking in some degree structural features of PN, were not inhibitory.

The virtual cessation of PN phosphorylation whenever the intraplatelet PNP approached 26 μM suggests product inhibition of PL phosphokinase by PNP. Such a control mechanism is consistent with previous studies in rabbit brain[18] wherein the concentration of pyridoxal phosphate controls the phosphokinase activity. Also, PLP appeared to be a noncompetitive inhibitor of PL phosphokinase from *Escherichia coli*.[19] Calculation of the total PLP and PMP concentration in cerebral cortex from the data of Loo and Mack[20] is in reasonable agreement with the 26 μM PNP in the platelet which apparently inhibits PL phosphokinase. Such a regulatory hypothesis is particularly attractive since the apparent diffusion rate of PN into the platelet is not limiting for phosphorylation.

Inhibition of PN phosphorylation by 4-DOP, PL and PM could be explained by one or both of the following: they may compete directly with PN for the phosphokinase or, themselves, become phosphorylated and impose product inhibition as previously described. Assuming 4-DOP, PL and PM have equal access as PN to the intraplatelet compartment, the phosphokinase appears to have a higher affinity for 4-DOP, PL and PM. The predominance of PL, PM and their phosphates in animal tissue,[21,22] and the fact that PL (and PM) is a more abundant source of the vitamin in the diet of auxotrophic organisms[23] are also consistent with the apparent "preference" of platelet phosphokinase for PL and PM. Similar to muscle,[24] the platelet has little if any PN or PNP oxidase.

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