The Uptake of Pyridoxal Phosphate by Human Red Blood Cells

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In order to examine whether pyridoxal phosphate could or could not be transferred into red blood cells in the form of a phosphate ester, the red blood cells were incubated with 14C-32P-pyridoxal phosphate and the following results were obtained.

1. The radioactivity of the extracellular medium decreased with time, and the same amount of radioactivity was found in the cell fraction. The ratio of 14C:32P in the medium or in the cells did not change throughout the reaction.

2. Most of the radioactivities present in the cells were recovered in the form of pyridoxal phosphate. The ratio of 14C:32P of pyridoxal phosphate in the cells was the same as that in the medium. A slight amount of radioactivity of 14C of pyridoxal and 32P of the inorganic phosphate were observed in the cells.

3. NaF and CH3COOH inhibited the transfer of pyridoxal phosphate to the extent of 50%. At low temperature, the transfer of the radioactivity into the cell was remarkably depressed.

4. After hemolysis, the radioactivity in the red blood cells was liberated in the supernatant. By starch-gel electrophoresis and Sephadex-gel filtration, pyridoxal phosphate was found to be combined with hemoglobin.

5. The red blood cells containing pyridoxal phosphate was incubated with hemoglobin solution, but pyridoxal phosphate was not liberated from the cells. The pyridoxal phosphate was not hydrolyzed by alkaline phosphatase. These results suggested that pyridoxal phosphate was not merely adsorbed on the surface of cell membrane, but was transferred through the cell membrane. These observations lead to the conclusion that pyridoxal phosphate was transferred into the red blood cells without being hydrolyzed to pyridoxal and inorganic phosphate.

Although many coenzyme forms of vitamins cannot penetrate cell membranes by simple passive diffusion, pyridoxal phosphate (PAL-P) is known to be taken up by Ehrlich ascites tumor cells and red blood cells (1,2). However, the characters of the uptake have not been elucidated yet. The mechanism of PAL-P uptake, therefore, is the focus of considerable interest and the object of our effort. Without any definite evidences, many investigators have considered the uptake to be a mere adsorption on the cytoplasmic membrane.

The present study aims to elucidate the mechanism of PAL-P uptake. The experiments have been carried out with human red blood cells and 14C-32P double labeled PAL-P in a...
Krebs-Ringer phosphate buffer.

These experiments resulted in proving the evidences of the direct uptake of the phosphate ester, denying the possibility of decomposition and resynthesis of PAL-P in transport. Furthermore, they also provided concrete evidences that the uptake meant transportation of the phosphate ester through the human erythrocyte membrane rather than a mere adsorption.

**Materials and Methods**

1. **Chemicals**

PAL-P was obtained from Chugai Pharmaceutical Company, Ltd., Tokyo. Pyridoxamine and intestinal alkaline phosphatase were purchased from Sigma Chemical Company. $^{14}$C-pyridoxine (PIN), i.e., 2-methyl-3-hydroxy-4,5-$\beta$-hydroxymethylpyridine-$\beta$-hydroxymethyl-$^{14}$C hydrochloride, was a gift of Dr. Wiss, Hoffmann La Roche Inc. $^{32}$P$_2$PO$_4$ was purchased from Radiochemical Centre, Amersham, England through the Japan Isotope Society. ATP and other chemicals were of the highest purity available from commercial sources.

2. **Preparation of $^{14}$C-PAL-P**

$^{14}$C-PAL-P was prepared enzymatically. The enzyme was prepared as described below. Freshly harvested Saccharomyces carlsbergensis was dried into a fine powder with acetone. Twenty-five grams of acetone powder were suspended in 75 ml of 0.1 M potassium phosphate buffer, pH 6.85, and treated in a Schlossman homogenizer (4,000 rpm for 1 min, twice). They were centrifuged at 10,000 rpm for 15 min. To 41 ml of the supernatant fluid, 10 g of ammonium sulfate was added and after 1 hour, it was centrifuged again. The precipitate was dissolved in 10 ml of 0.1 M potassium phosphate buffer, pH 6.85. To 10 ml of enzyme solution were added 2.5 mg of FMN, 0.25 ml of 0.1 M MgSO$_4$, 25 mg of ATP and 8 x $10^7$ cpmp of $^{14}$C-pyridoxine (specific activity, 30.8 $\mu$Ci/mg). The pH of the mixture was adjusted to 10.0. The incubation was carried out at 37$^\circ$C for 2 hours. The reaction was stopped by adding 10 ml of 30% perchloric acid and then centrifuged. The supernatant was neutralized to pH 7.0 with KOH and the precipitated KClO$_4$ was removed by centrifugation. The solution obtained was passed through a column of Dowex-50W-H$^+$ (3 x 50 cm), and the column was washed with 150 ml of water and then with 1 N NaOH. The pH of the effluent changed from acid to neutral and then to alkaline. Neutral and alkaline fractions were collected, condensed, and applied on the Whatman No. 3 MM filter paper and developed with a solvent system of ethylacetate-n-propanol-water-diethylamine (80:60:50:10). The area of pyridoxine (RF 0.80) and PAL-P (RF 0.25) were eluted out with water and the pyridoxine recovered was used repeatedly for the preparation of $^{14}$C-PAL-P. PAL-P was then placed on a column of Amberlite CG-50 (H$^+$) and eluted with water. A typical procedure gave a yield of about 3.5 x $10^6$ cpm of PAL-P.

3. **Preparation of $^{32}$P-PAL-P**

$^{32}$P-PAL-P was prepared from $^{32}$P$_2$PO$_4$ and pyridoxamine by a slight modified method of Kuroda [3]. One hundred milligrams of pyridoxamine hydrochloride, 5 mCi of $^{32}$P$_2$PO$_4$ in 0.05 ml of 0.1 N HCl and 200 mg of P$_2$O$_5$ were mixed well. The mixture was placed in a desiccator with P$_2$O$_5$ for three days at 30$^\circ$C and then dissolved in 2 ml of cold water. This solution was put onto a column (3 x 50 cm) of Dowex-50W-H$^+$. The column was washed with 250 ml of deoxygenized water and then with 100 ml of 1 N NH$_4$OH. The alkaline fraction, which contained both pyridoxamine (PAM) and PAM-P, was collected and condensed to 5 ml. Into the mixture of PAM and PAM-P, 400 mg of colloidal MnO$_2$ were added and the reaction was allowed to proceed for about 2 hours at 20$^\circ$C. At the end of the period the ninhydrin reaction of the mixture was almost negative. The mixture was filtered and the filtrate was immediately passed through a column of Ambelite CG-50-H$^+$. The column was washed with deoxygenized water. The effluent was condensed under a reduced pressure and then subjected to paper chromatography by an ascending method with the solvent of ethylacetate-n-propanol-water-diethylamine as shown above. The area of PAL-P was eluted with water and the effluent was condensed. The solution was placed on the column of Dowex-1 x 8 (acetate form) and eluted with 0.3 N acetic acid. After extraction of excess acetic acid with ether, PAL-P was placed on a column (1 x 20 cm) of Dowex-50 W-H$^+$ and eluted with 1 N NH$_4$OH. The yield of $^{32}$P-PAL-P was about 68 mg and its total count was 5 x $10^6$ cpm.

4. **Preparation of $^{14}$C-, $^{32}$P-PAL-P**

$^{14}$C-PAL-P and $^{32}$P-PAL-P were mixed and used as the double labeled PAL-P. Usually 15 x $10^4$ cpm of $^{14}$C-PAL-P and 12.5 x $10^4$ cpm of $^{32}$P-PAL-P were employed on one experiment.

A fresh colloidal MnO$_2$ was prepared as follows: 0.45 g of KMnO$_4$ was reduced with Na$_2$SO$_3$ and MnO$_2$ thus formed was absorbed with 2 g of cellulose powder.
5. Measurement of Radioactivity
The radioactivity was measured by a Packard Liquid Scintillation Spectrometer with 10 ml of Bray’s solution (4). The counts of $^{32}$P and $^{14}$C were measured at 700 and 1080 V respectively. All the counts at 700 V were due to $^{32}$P and the ratio of $^{14}$C: $^{32}$P at 1080 V was 85:15 respectively.

6. Measurement of PAL-P and PAL
PAL-P and PAL were spectrophotometrically measured by the phenylhydrazine method using a Bausch and Lomb spectrophotometer (5). For the determination of PAL-P, the sample was kept at 0°C for 20 min after adding 0.5 ml of 18 N H$_2$SO$_4$ and 0.2 ml of 2 % phenylhydrazine. By this procedure PAL-P was quantitatively changed to hydrazine derivative, while PAL could be detected after heating at 60°C for 30 minutes.

7. Preparation of Washed Red Blood Cells
One hundred milliliters of heparinized blood from a healthy man was centrifuged and the fluffy layer of white blood cells was removed as far as possible. The red blood cells thus obtained were washed with saline solution twice and finally with Krebs-Ringer phosphate solution containing 0.1% glucose. Total cell volume was 42.5 ml. The cells were suspended in an adequate volume of Krebs-Ringer phosphate solution to obtain a hematocrit value of 50%.

8. Preparation of Ghost Cells
The red blood cell membrane was prepared by the method of Post (6). Six milliliters of fresh red blood cells were washed with 50 ml of 0.15 M NaCl twice. After hemolysis with 30 ml of cold distilled water, they were centrifuged and washed with 50 ml of 5 × 10$^{-4}$ M histidine-imidazole buffer repeatedly. Resulted faint pink precipitate was used as the cell membrane preparation.

9. Transfer of PAL-P into the Red Blood Cells
A mixture of 10.1 ml containing 1 mM radioactive PAL-P (15 × 10$^4$ cpm of $^{14}$C, 12.5 × 10$^4$ cpm of $^{32}$P) and red blood cells was incubated at 37°C. Aliquots of 2 ml each were taken at time intervals and washed with 50 ml of 0.9% NaCl solution three times. By repeated washings, the radioactivity originally present in the medium was nearly removed from the cells, as shown in Table 1. Radioactivities of the washings were measured directly with Bray’s solution and those of the cells were counted in trichloroacetic acid extracts.

10. Isolation and Detection of $^{14}$C.$^{32}$P-PAL-P from the Red Blood Cells
The incubation mixture was deproteinized by addition of trichloroacetic acid. After the removal of the acid by several ether extractions, the solution was placed on a column (1 × 30 cm) of Dowex-50 (H$^+$ form). It was washed by 50 ml of water and then by 75 ml of 0.1 N NH$_4$OH. The purification procedure on a Dowex-50W column was necessary to remove contaminating ions which would interfere with the subsequent paper chromatography. Counts of inorganic phosphate, which was not adsorbed and eluted in the acidic fraction, were less than 10% of the applied counts. Neutral and alkaline fractions were collected and condensed by a flash evaporator. They were applied on the Whatman No. 1 filter paper and developed with the solvent of ethylacetate-$n$-propanol-water-diethylamine (80:60:50:10) for 12 hours.
hours in the dark. The filter paper was cut into 1 cm strips and their radioactive counts were measured directly.

RESULTS

1. Uptake of PAL-P by the Red Blood Cells

$^{32}$P,$^{14}$C-PAL-P at a final concentration of $10^{-4}$M was incubated with 10 ml of red blood cells at $37^\circ$ in the Krebs-Ringer phosphate solution. As shown in Fig. 1, counts of $^{14}$C and $^{32}$P in the medium decreased with time, whereas counts in the red blood cells increased. After 40 min incubation, 5% of total PAL-P was transferred to the cells. The ratio of $^{14}$C:$^{32}$P of the radioactivity recovered from the cell fraction was the same as that of the labeled PAL-P added to the medium.

![Graph](image)

**FIG. 1 Transfer of $^{14}$C,$^{32}$P-PAL-P from incubation medium to the red blood cells**

The red blood cells were suspended in the Krebs-Ringer phosphate solution, pH 7.4, containing 0.1% of glucose. One mmole of double labeled PAL-P (radioactivity of $^{14}$C was 150,000 cpm and $^{32}$P was 125,000 cpm) was added to 10 ml of the red blood cells. The suspension was incubated at $37^\circ$ for 0, 10, 20, 40 or 60 min. 2 ml of the reaction mixture were washed three times with 50 ml of 0.9% NaCl solution. Radioactivities of cells and washings were counted $\times - \times$, $^{32}$P; $\circ \cdots \circ$, $^{14}$C.

2. Identification of Radioactive Materials in the Cells

$15 \times 10^4$ cpmp of $^{14}$C- and $12.5 \times 10^4$ cpmp of $^{32}$P-PAL-P were incubated with 10.1 ml of red blood cells for 60 min at $37^\circ$. At the end of the incubation period, 2 ml of 30% trichloroacetic acid were added to the incubation mixture. Coagulated protein was removed by centrifugation. The clear supernatant fluid was condensed and developed by paper chromatography as described above. As shown in Fig. 2, more than 70% of radioactive $^{14}$C and $^{32}$P were detected as PAL-P. Moreover, the ratio of $^{14}$C and $^{32}$P was the same as that of the initial PAL-P. Only a slight amount of PAL-P was decomposed to inorganic phosphate and PAL.

![Graph](image)

**FIG. 2 Paper chromatography of $^{14}$C,$^{32}$P-PAL-P recovered from the red blood cells**

150,000 cpm of $^{14}$C and 125,000 cpm of $^{32}$P-PAL-P were incubated with 10 ml of the red blood cells at $37^\circ$ for 60 min. The cells were isolated and deproteinized. The extract was developed by paper chromatography as described in the text. Counts of $^{14}$C were corrected for counting efficiency. $\sim$, $^{32}$P; $\cdots$, $^{14}$C.

### Table 3

<table>
<thead>
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<th>Incubation time (min)</th>
<th>0</th>
<th>20</th>
<th>40</th>
<th>60</th>
</tr>
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<tbody>
<tr>
<td>PAL-P ($\mu$moles)</td>
<td>0.004</td>
<td>0</td>
<td>0</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Uptake of PAL-P by the same amount of hemoglobin was higher than 0.100 $\mu$ mole.

Dry weight of cells was 67.0 mg.

Ghost cells prepared from 6 ml of blood were incubated with $10^{-3}$M PAL-P at $37^\circ$. They were washed with 0.9% NaCl solution, and PAL-P uptaken by ghost cells was measured by the phenylhydrazine method.
3. Effect of Temperature on the Uptake of PAL-P by the Cells

The mixture of the radioactive PAL-P and the red blood cells was incubated for 60 min. Incorporation of PAL-P into the red blood cells at ° was less than 10% of that at °, as shown in Fig. 3. This fact suggests that the uptake of PAL-P by the cells is not due to the mere adsorption on the membrane but possibly due to the transportation into the cells.

4. Inhibition of Uptake by NaF and CH$_2$COOH

NaF and CH$_2$COOH were added to the incubation mixture at a final concentration of 10$^{-3}$M. As shown in Fig. 4, the uptake of radioactivity of PAL-P was depressed by less than 50% of the original. This fact also suggests that the uptake of PAL-P is the transportation into the cells.

5. Localization of PAL-P in the Cells

The cells incubated with PAL-P were washed with 50 ml of 0.9% NaCl solution three times. To 2 ml of the washed red blood cells, 1 ml of distilled water was added.
FIG 6 Sephadex gel filtration of hemoglobin PAL-P complex

1 ml of hemoglobin PAL-P complex was placed on a Sephadex G-25 column (2.5 x 30 cm) and eluted with 0.01 M phosphate buffer, pH 7.4. Fraction of PAL-P was detected spectrophotometrically by the phenylhydrazine method.

0.1 ml of the supernatant was placed on the starch gel (1.5 x 20 cm) and the electrophoresis was carried out for 4 hours at pH 8.4 (7). Upper half of the gel was cut into 1 cm strips. After hydrolysis with 1 N HCl, amounts of pyridoxal in each fractions were assayed biologically by Saccharomyces carlsbergensis 4228 ATCC 9080 (8). Hemoglobin in the lower half of the gel was stained with amido-black 10-B. As shown in Fig. 5, the PAL fraction agreed with the hemoglobin fraction.

The same result was obtained by the experiment of Sephadex G-25 gel filtration. One milliliter of the supernatant was placed on a Sephadex G-25 column (2.5 x 30 cm), and it was eluted with 0.01 M of phosphate buffer, pH 7.4. PAL-P and hemoglobin was eluted in the same fraction but neither liberated PAL-P nor PAL was detected (Fig. 6).

6. Retention of PAL-P into the Red Blood Cells in Spite of Washings with Hemoglobin

The cells incubated with PAL-P were washed three times with 50 ml of 0.9 % cold NaCl solution. As shown in Table 1, PAL-P was not liberated in the washing medium and a great part of PAL-P remained in the cells. Even when these cells containing PAL-P were incubated again with an isotonic solution of hemoglobin at 37° for 30 min, PAL-P still remained in the cells without being found in the added hemoglobin fraction. These results suggest that PAL-P was not merely adsorbed on the surface of cell membrane (Table 2).

7. Absorbability of Ghost Cells

Ghost cells prepared from 6 ml of blood by the method of Post (6) were incubated with 10^{-8} M PAL-P at 37° for 30 min. After centrifugation a large part of PAL-P was found in the supernatant. Washings with 0.9 % of NaCl solution left no PAL-P to be detected on the ghost cells. In this period, only 12 per cent of PAL-P was hydrolyzed to PAL and inorganic phosphate. This result suggested that cell membranes had no ability of adsorbing PAL-P (Table 3).

8. Decomposition of PAL-P by Alkaline Phosphatase

Ten milligrams of intestinal alkaline phosphatase was incubated with red blood cells

FIG. 7 Hydrolysis of PAL-P by alkaline phosphatase

10 mg of an intestinal alkaline phosphatase was incubated with red blood cells containing PAL-P or other substrate at pH 7.4. Remaining PAL-P was measured spectrophotometrically with phenylhydrazine method.
containing PAL-P. To avoid the hemolysis of red blood cells, the incubation was carried out at pH 7.4, which was not optimal pH for the alkaline phosphatase. Even at this pH, enzyme hydrolyzed either PAL-P or PAL-P hemoglobin complex pretty well. As shown in Fig. 7, they were hydrolyzed about 60-75% in 30 min. On the other hand, PAL-P taken by the red blood cells was hydrolyzed only 15% in 30 min. This result also indicated that PAL-P was not merely adsorbed on the surface of red blood cells.

9. Comparison of the Rate of Transfer of PAL-P into the Red Blood Cells with that of Free Pyridoxal

As shown in Fig. 8, 30% of PAL was detected in the cells after 60 min incubation at 37°, whereas only 5% of PAL-P were detected in the cells.

DISCUSSION

The mechanisms of the transport of amino acids, sugars and inorganic ions across biological membranes have been a focal point of interest of numerous investigators; however, the transport of phosphate esters has not been studied so extensively. Only some hexose phosphates and D-α-glycerophosphate were thought to be actively transported through the plasma membrane of Escherichia coli (9, 10). Moreover, it was believed without a concrete evidence that phosphate compounds were very hard to transfer across the cell wall. But many bacterial strains which excrete PAL-P into the medium were now available (11, 12). Accumulation of PAL-P in the culture medium suggests that PAL-P may pass through the bacterial cell as the phosphate ester. This experiment has provided some evidences for the possibility of the transportation of phosphate esters across the cell membrane.

The red blood cell has a phosphatase activity which does not appear in the fresh non-hemolytic state (13), but does appear when it is hemolyzed. In this experiment, the hemolyzed red blood cells hydrolyzed PAL-P to the extent of 25% at 37° for 1 hour.

Effects of temperature and inhibitors were remarkable. Evidences presented in these experiments support the view that PAL-P is not merely adsorbed on the cell membrane, but transfers into the cells.

Further evidences of the transport were given by the experiments of the deprivation of PAL-P from intact cells by adding high concentration of hemoglobin, a failure of adsorption of PAL-P by the ghost cell membrane, and a loss of hydrolyzing activity to PAL-P taken into red blood cells by alkaline phosphatase. These evidences also confirm that PAL-P penetrates across the blood membrane.

Recently Tsuji and Yamada showed that the concentration of PAL-P in the red blood cells did not increase whatever the concentrations of PIN, PAL or PAM increased in the reaction mixture. However, when PAL-P was added, its concentration in erythrocytes increased proportionally to the added PAL-P concentration. Moreover, inorganic phosphate ion in the medium increased the rate of the transfer of PAL-P, in spite of the fact that the phosphate ion was a strong inhibitor of
blood phosphatase (14). This suggests that PAL-P was directly transferred into the red blood cells without any hydrolysis.

REFERENCES