

# Conversion of Vitamin B<sub>6</sub> Compounds to Active Forms in the Red Blood Cell

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**ABSTRACT** In studies with pyridoxine and other B<sub>6</sub> compounds in blood, the active forms pyridoxal and pyridoxal phosphate were measured by differential assays using *Lactobacillus casei*. Red cell uptake of tritiated pyridoxine was also measured. A new metabolic pathway for conversion of pyridoxine to active forms was demonstrated in red cells.

In vivo studies in normal subjects suggested that pyridoxine was taken up by red cells where it was converted to pyridoxal phosphate and then pyridoxal, followed by gradual release of a proportion of pyridoxal into plasma. In vitro incubation of pyridoxine with blood confirmed this observation.

Increasing amounts of pyridoxine were taken up and converted as the amount added to blood was increased, and only very small numbers of red cells were needed to convert appreciable amounts. Conversion was markedly inhibited at temperatures lower than 37°C, and stopped altogether at -20°C.

Release of pyridoxal into plasma was always directly proportional to the amount of pyridoxal formed and to the volume of plasma present. That pyridoxal phosphate was not released into plasma was demonstrated in stored blood, for pyridoxine was converted mainly only as far as pyridoxal phosphate, probably due to inactivation of the phosphatase. Pyridoxal phosphate remained in the red cells.

Pyridoxine was converted when incubated with washed red cells in saline or phosphate buffer suspension (0.08 M). In saline suspension, pyridoxal formed but was not released in the absence of plasma. In phosphate buffer suspension, pyridoxal phosphate was formed but was not changed to pyridoxal, probably due to inactivation of phosphatase by excess phosphate.

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Pyridoxamine was converted to active forms in red cells less efficiently. Pyridoxal entered red cells rapidly, equilibrating between plasma and cells within 1 min in the same ratio as pyridoxal formed inside red cells. Pyridoxal phosphate did not enter red cells in whole blood but did so readily in washed cells in saline.

## INTRODUCTION

There are three forms of vitamin B<sub>6</sub>; pyridoxine (the alcohol), pyridoxamine (the amine), and pyridoxal (the aldehyde). These can also exist in the phosphorylated form. Pyridoxal phosphate is known to be the main active coenzyme form and it acts as such in many enzyme systems in the body.

Pyridoxamine and pyridoxal predominate in meat and other animal products, but in fruit, vegetables, and cereals pyridoxine is the main form (1-4). It is apparent that pyridoxine must be an important form of B<sub>6</sub> for many individuals and it has also been most commonly used for therapy. The mode and site of conversion of pyridoxine to forms active in the body is therefore of considerable biological and clinical interest.

Exactly how and where the conversion of pyridoxine and other B<sub>6</sub> compounds takes place in the body has not been fully established. The pathway usually postulated for pyridoxine is by conversion first to pyridoxine phosphate and then to pyridoxal phosphate, the conversions being activated by the appropriate enzymes, a kinase and oxidase respectively. This reaction has been demonstrated in vitro in liver homogenates (5), but whether this is the essential or sole site of conversion is doubtful.

We measured the appearance of pyridoxal and pyridoxal phosphate in blood at varying time intervals after oral administration of pyridoxine. The microbiological assay method with *Lactobacillus casei* was used (6). We found that there was a rapid conversion of pyridoxine to these forms, and this led us to carry out in

vitro studies in which pyridoxine and other forms of B<sub>6</sub> were incubated with blood under varying conditions. The red cell was found to play an important role.

## METHODS

All blood samples were taken from healthy members of the staff and were heparinized (125 IU lithium heparin per 10 ml). Blood and B<sub>6</sub> compounds were protected from light throughout (6).

The unlabeled B<sub>6</sub> compounds were obtained from Sigma Chemical Co. (98–99% crystalline) except pyridoxine-5'-phosphate which was obtained from Mann Research Labs, Inc. Tritium-labeled pyridoxine (Pyridoxine-T(G) Hydrochloride) was obtained from the Radiochemical Centre, Amersham, England.

### Experimental procedure

*In vivo studies.* A 3-ml blood sample was taken from a subject who had fasted overnight. An oral dose of 50 mg of pyridoxine HCl was then given with water and no food was taken for 3 hr. 3-ml blood samples taken at intervals from 10 min up to 24 hr afterwards, were plunged immediately into ice and prepared for storage as described below.

*In vitro studies.* In the form of pyridoxine HCl, the required amount of pyridoxine (50–2000 ng) in 0.01 ml of 0.15 M NaCl was added per ml of blood. The same conditions applied to other forms of B<sub>6</sub>. Tritium-labeled pyridoxine (pyridoxine-<sup>3</sup>H) was added for measurement of red cell uptake and the specific activity was always adjusted so that 0.005  $\mu$ Ci was added per ml of blood.

Whole blood (or red cell suspension), and apparatus were equilibrated to 37°C in a warm room. In every experiment a base line sample was kept for assay. B<sub>6</sub> was added to the required amount of blood within 2 hr of being drawn, then 3-ml portions were put in stoppered conical flasks and were incubated in a shaking waterbath at 37°C at 70 strokes per minute. The portions were removed at intervals up to 3 hr, plunged immediately into ice, and prepared for storage as described below.

### Subsequent treatment and storage of blood

To stop enzyme activity at the end of each time interval, about 0.3 ml of whole blood (or red cell suspension) was immediately deep frozen to –20°C. Plasma (or suspending fluid) was separated immediately from the remaining blood at 4°C and deep frozen to –20°C.

For assay of red cells, plasma from 1 ml of blood was removed immediately at 4°C, cells were washed three times in cold 0.15 M NaCl, the volume was made up to 1 ml with cold 0.15 M NaCl, and cells were deep frozen to –20°C.

### Measurement of B<sub>6</sub> compounds

*Measurement of pyridoxal and pyridoxal phosphate.* The assay organism *Lactobacillus casei* NCIB 8010 (ATCC 7469) was used as described for the assay of serum by Anderson, Peart, Fulford-Jones (6). *L. casei* measures only pyridoxal directly and responds to 0.01 ng per ml. To measure pyridoxal phosphate it must first be hydrolyzed to pyridoxal. Neither pyridoxine nor any of the other known forms of B<sub>6</sub> can be measured at the dilutions used, whether hydrolyzed or not.

Whole blood (or red cell suspension) and plasma (or suspending fluid) were assayed.<sup>1</sup> The red cell value was calculated and it was confirmed by direct assay that this was valid. Errors due to washing cells and the delay involved were thus avoided. Specimens were pipetted as soon as they thawed to prevent resumption of enzyme activity, and before assay, were treated as follows: 0.1–0.0025 ml was (a) autoclaved in 0.2 N HCl at 121°C for 1 hr, brought to pH 6.4 and filtered (6) and (b) diluted in distilled water. The final volume in either case was 20 ml and the final dilution in the assay varied from 1 in 500 to 1 in 20,000. The following measurements could be made: In (a) pyridoxal plus pyridoxal phosphate, referred to as *L. casei* active B<sub>6</sub>, was measured as pyridoxal in the filtrate. In (b) pyridoxal was measured. The pyridoxal phosphate value was calculated from the difference between (a) and (b).

In the *in vivo* studies, the increase of *L. casei* activity over the endogenous level<sup>2</sup> was calculated for 100 ml of whole blood and for the plasma and red cells from 100 ml of blood. This was expressed as  $\mu$ g per 100 ml of blood.

In the *in vitro* studies, as the molecular weight of pyridoxal and pyridoxine (or pyridoxamine) are practically the same, the increase in *L. casei* activity (difference between test and base line levels) was most conveniently expressed as a percentage of the added pyridoxine (or pyridoxamine), but in some experiments was expressed as nanograms. The conversion (see Results) refers to the measurement of pyridoxal phosphate plus pyridoxal in whole blood.

*Measurement of tritium-labeled B<sub>6</sub>.* Tritium-labeled B<sub>6</sub> in plasma was measured in a Packard liquid scintillation counter (model 3375) and was compared with the activity in internal standards. To do this, 0.2 ml of plasma was digested in 1 ml of hyamine overnight before addition of scintillating fluid and subsequent counting.

The red cell uptake of pyridoxine-<sup>3</sup>H was calculated from the tritium measurement in plasma because there could be errors in measuring the activity in red cells due both to the effects of washing cells and to quenching of radioactive counts. The uptake at any time was the difference between the added pyridoxine-<sup>3</sup>H and that remaining in plasma, but account had to be taken of pyridoxine which had entered cells and then been released into plasma as tritiated pyridoxal (see Results). Therefore, pyridoxine which had remained in plasma was the difference between total tritiated B<sub>6</sub> activity in plasma and the increase in *L. casei* activity in plasma. The percentage of total pyridoxine which had been taken up by red cells by any one time was calculated from the following formula:

$$100 - \frac{5A(100-PCV)}{S} + B$$

where A = counts per minute in 0.2 ml of plasma (pyridoxine + pyridoxal)

B = increase in *L. casei* active B<sub>6</sub> in plasma as per cent of added pyridoxine

S = counts per minute in 500 ng of pyridoxine-<sup>3</sup>H in 0.2 ml of plasma.

<sup>1</sup> Reproducibility, the combined coefficient of variation was 10.4% when seven control plasmas (80–340 ng/ml) were assayed between 10 and 40 times in consecutive assay batches (7).

<sup>2</sup> Pyridoxal phosphate plus pyridoxal concentration in control subjects used: 9–17 ng/ml in whole blood; 4–14 ng/ml in plasma.

## RESULTS

### In vivo studies

#### ORAL INGESTION OF 50 MG OF PYRIDOXINE HCL

*Appearance of *L. casei* active  $B_6$  in blood.* Increased *L. casei* active  $B_6$  was found in hydrolyzed blood after an oral dose of pyridoxine (Fig. 1A). Pyridoxine had been rapidly absorbed, for at 10 min there was increased *L. casei* activity in the blood equivalent to 1.6  $\mu\text{g}/100\text{ ml}$ , all of which was in the red cells. The activity increased rapidly to a peak at 1 hr and was mainly in the red cells. A second unexplained peak was frequently found at 2 hr. At 20 min there was activity in the plasma which gradually increased, reaching a plateau between 1 and 2 hr, and then decreased.

At least part of the absorbed pyridoxine had been converted to *L. casei* active  $B_6$ . The accumulation in the red cells suggested that this conversion might take place in the red cells.

*Forms of *L. casei* active  $B_6$ .* There was both pyridoxal and pyridoxal phosphate in red cells but in plasma there appeared to be only pyridoxal (Fig. 1B).

### In vitro studies

#### INCUBATION OF PYRIDOXINE WITH BLOOD (500 NG/ML)

*Conversion to *L. casei* active  $B_6$ .* When pyridoxine was incubated with blood *L. casei* active  $B_6$  was formed as was found after oral ingestion of pyridoxine (Fig. 2A). At 1 min, 7% had been converted and was entirely in the red cells which added support to the suggestion that conversion was carried out by the red cells. The *L. casei* activity increased rapidly and by 1 hr 95% had been converted.

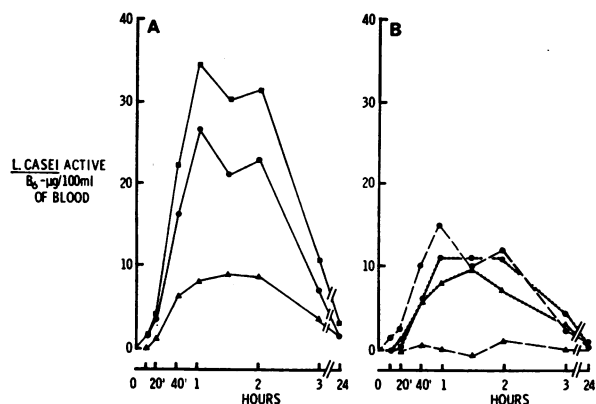


FIGURE 1 Increase in *L. casei* active forms of  $B_6$  in whole blood, red cells, and plasma after oral ingestion of 50 mg of pyridoxine HCl. Whole blood, ■; Red cells, ●; Plasma, ▲; —, Pyridoxal phosphate + pyridoxal; —●—●—●—, Pyridoxal; — — —, Pyridoxal phosphate.

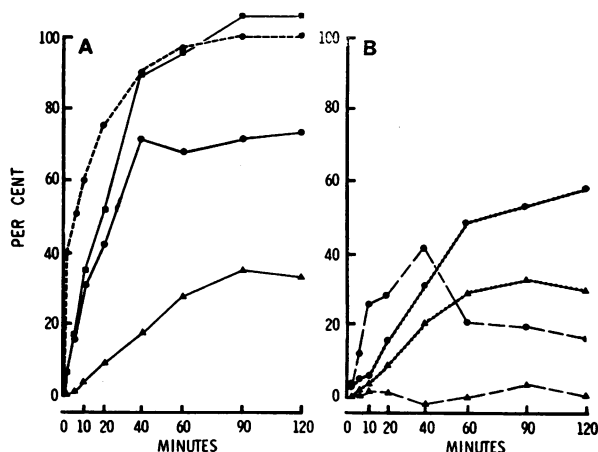


FIGURE 2 Increase in *L. casei* active forms of  $B_6$  in whole blood, red cells, and plasma during incubation of pyridoxine with blood (500 ng/ml). (Shown on abscissa as *L. casei* active  $B_6$  as per cent of added pyridoxine). Whole blood, ■; Red cells, ●; Plasma, ▲; —, Pyridoxal phosphate + pyridoxal; —●—●—●—, Pyridoxal; — — —, Pyridoxal phosphate; — — —, Total red cell uptake of pyridoxine (see Methods).

In plasma there was a trace of activity by 5 min which gradually increased to a plateau of 35% at 90 min. This steady increase in the plasma after the appearance of activity in the red cells, and reflecting the picture found after oral ingestion of pyridoxine, suggested that some of the activity formed in the red cells was gradually released into plasma. There was no hemolysis. If pyridoxine was incubated with plasma, conversion to *L. casei* active  $B_6$  did not occur.

*Forms of *L. casei* active  $B_6$ .* Initially, the main form in red cells was pyridoxal phosphate (Fig. 2B). The amount of pyridoxal gradually increased and after 40 min pyridoxal phosphate decreased, so that by 1 hr a large proportion was pyridoxal. As before, only pyridoxal could be detected in plasma.

*Red cell uptake of pyridoxine.* Uptake of pyridoxine- $^3\text{H}$  was 40% by 1 min (Fig. 2A), but was not complete until 90 min. The conversion lagged behind, for only 7% was converted by 1 min. However, by 40 min all pyridoxine that had been taken up was converted.

#### INVESTIGATION OF FACTORS INFLUENCING METABOLISM OF PYRIDOXINE IN BLOOD

*Volume of red cells.* The volume of red cells was artificially adjusted from a sample of blood to give a series in which PCV<sup>3</sup> varied from 1 to 59%. 500 ng of pyridoxine- $^3\text{H}$  per ml was incubated with each sample for 60 min.

The percentage conversion of pyridoxine to pyridoxal phosphate plus pyridoxal is plotted against the PCV

<sup>3</sup> Abbreviations used in this paper: PCV, packed cell volume.

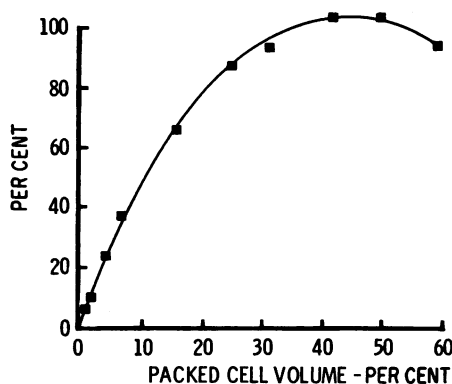


FIGURE 3 Conversion to *L. casei* active B<sub>6</sub> (pyridoxal phosphate plus pyridoxal) after incubation of pyridoxine (500 ng/ml) for 60 min with samples of the same blood adjusted to contain increasing volumes of red cells. (Shown on abscissa as total *L. casei* active B<sub>6</sub> as per cent of added pyridoxine).

(Fig. 3). There was a significant conversion of 6% when the PCV was only 1% and as much as 37% (175 ng/ml) when the PCV was 7%. The conversion increased up to a PCV of 43% but for a PCV of 59% there was a slight inhibition. The red cell uptake did not exceed the conversion and appeared to be the limiting factor.

**Amount of pyridoxine added.** Amounts of 50–2000 ng of pyridoxine-<sup>3</sup>H were incubated per ml of blood for times varying from 5 min to 2 hr, and the red cell uptake of pyridoxine and conversion to pyridoxal phosphate plus pyridoxal were measured (Fig. 4).

**UPTAKE:** As the amount of added pyridoxine was increased larger amounts were taken up by the cells. The proportion in relation to the added amounts only de-

creased slightly. A large proportion entered the cells within the first 5 min, but a slower uptake followed.

**CONVERSION:** At first, the conversion lagged behind the uptake as shown previously, but by 2 hr even the main part of that taken up from 2000 ng was converted.

**Pyridoxal formation and plasma volume.** The appearance of pyridoxal in plasma was related to at least two factors:

1. **AMOUNT OF PYRIDOXAL FORMED IN BLOOD:** Increasing amounts of pyridoxine (50–2000 ng/ml) were incubated with samples of the same blood for times varying from 20 min to 2 hr. The amount of pyridoxal released into plasma bears a direct linear relationship to the amount of pyridoxal formed in blood (Fig. 5), and not to the total conversion to pyridoxal phosphate plus pyridoxal. The mean percentage of pyridoxal which appeared in the plasma was 33% of the total pyridoxal formed in blood.

2. **THE AMOUNT OF PLASMA PRESENT:** Increasing amounts of plasma removed from samples of the same blood were replaced by equal volumes of 0.15 M NaCl. Thus, the amount of red cells and total volume were constant while the amount of plasma present in each sample decreased. 500 ng of pyridoxine per ml was incubated with each sample and approximately the same amount was converted to pyridoxal in each. There is a direct linear relationship between the amount of pyridoxal appearing in the plasma and the amount of plasma present (Fig. 6).

In addition, plasma was removed from whole blood after incubation of 500 ng of pyridoxine per ml for 90 min, in which circumstances there was maximum release of pyridoxal into plasma (see Fig. 2A). Fresh plasma

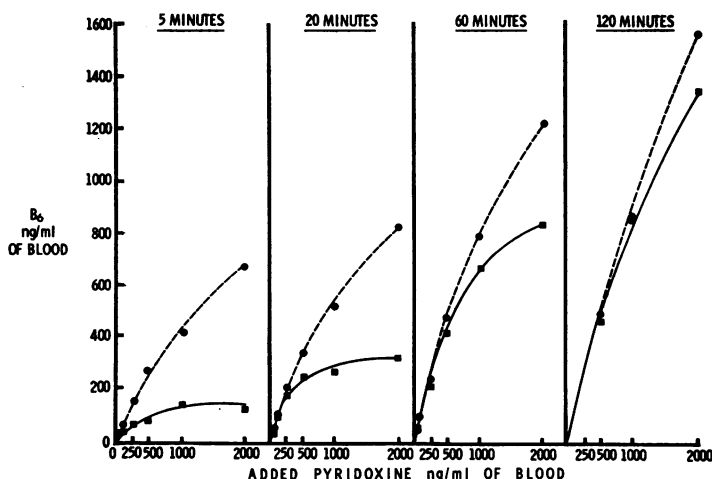


FIGURE 4 Red cell uptake of different amounts of pyridoxine-<sup>3</sup>H incubated with blood for varying lengths of time, and the conversion to *L. casei* active B<sub>6</sub>. ●—●, Total red cell uptake of pyridoxine; ■—■, Pyridoxal phosphate plus pyridoxal in whole blood.

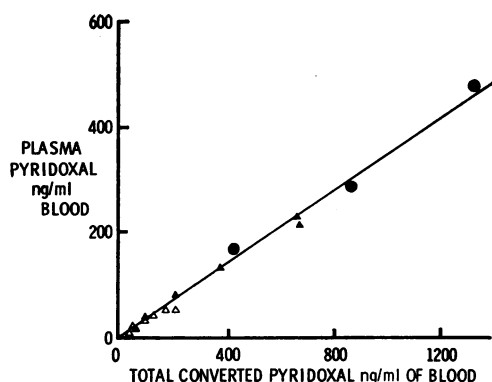


FIGURE 5 Relationship of amount of pyridoxal passing into plasma with amount of pyridoxal formed after incubation of different amounts of pyridoxine (50, 100, 250, 500, 1000, and 2000 ng/ml) with blood for varying lengths of time. Incubated for 20 min  $\Delta$ ; 60 min  $\blacktriangle$ ; 120 min  $\bullet$ .

was added and after a further incubation for 60 min an amount of pyridoxal relative to that in the red cells was again released into the plasma. On the other hand, when pyridoxine was incubated with washed cells suspended in 0.15 M NaCl there was practically no release of pyridoxal into saline, in spite of the fact that a major proportion was converted to pyridoxal.

**Temperature.** 500 ng of pyridoxine- $^3\text{H}$  per ml of blood was incubated at temperatures of 37, 22, and 4°C for times up to 60 min. The uptake of pyridoxine into cells, the conversion to pyridoxal phosphate plus pyridoxal and the release of pyridoxal into plasma were all decreased by lowering the temperature of incubation. At 4°C there was very marked inhibition with practically no release into plasma.

However, conversion of pyridoxine continued slowly at 4°C and 73% had been converted in 24 hr. There was no conversion at -20°C.

**Storage of blood.** Portions of blood were kept for periods up to 7 days, some on the bench at room temperature (23°C) and others at 4°C, and conversion to pyridoxal phosphate and pyridoxal was measured when the stored blood was incubated with pyridoxine (500 ng/ml) for 60 min at 37°C (Fig. 7).

**CONVERSION:** The total conversion to *L. casei* active  $\text{B}_6$  remained normal in blood which had been kept at 4°C, and up to 48 hr at 23°C. The longer that blood had been stored up to 48 hr the greater was the proportion of pyridoxal phosphate in the cells after incubation with pyridoxine, with a consequent reduction in pyridoxal. This was very marked in blood stored at 23°C.

**RELEASE:** The amount of pyridoxal appearing in plasma after conversion of pyridoxine decreased in blood that had been kept longer than 5 hr. The decrease was rapid in blood stored at 23°C and generally was in-

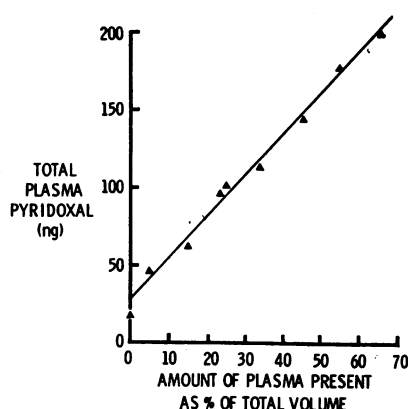


FIGURE 6 Relationship of amount of pyridoxal passing into plasma with amount of plasma present, after incubation of pyridoxine (500 ng/ml) for 60 min with samples of the same blood adjusted to contain increasing amounts of plasma. (Red cell volume constant and total volume kept constant with 0.15 M NaCl).

directly proportional to the accumulation of pyridoxal phosphate in the red cells and paralleled the decreasing amounts of pyridoxal. This observation suggests that the phosphate form of pyridoxal does not pass out of the cell.

**Washed red cells, intact and hemolyzed.** 500 ng of pyridoxine per ml was incubated for 60 min with (a) whole blood and (b) twice washed red cells suspended either in 0.15 M NaCl or in phosphate buffer in 0.15 M NaCl (pH 7.4) of varying molarity (0.01 M to 0.08 M), the concentration of cells being the same as that of whole blood. Conversion to *L. casei* active forms is illustrated in Fig. 8. The total conversion was approximately the same throughout, but practically no pyridoxal passed into the suspending fluid. The proportion of

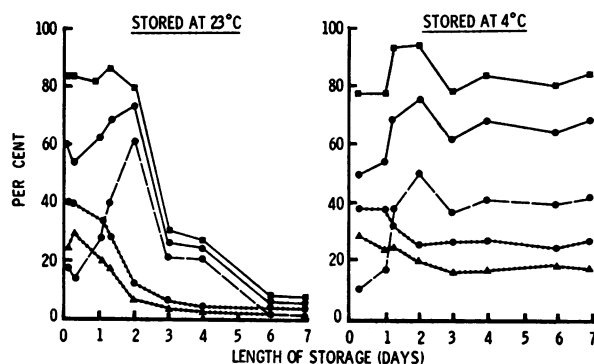


FIGURE 7 Effect of storage of blood before incubation with pyridoxine (500 ng/ml) on conversion to *L. casei* active forms of  $\text{B}_6$  and release into plasma after incubation for 60 min. (Shown on abscissa as *L. casei* active  $\text{B}_6$  as per cent of added pyridoxine). Whole blood,  $\blacksquare$ ; Red cells,  $\bullet$ ; Plasma,  $\blacktriangle$ ; —, Pyridoxal phosphate + pyridoxal;  $\circ$ — $\circ$ , Pyridoxal; — — — Pyridoxal phosphate.

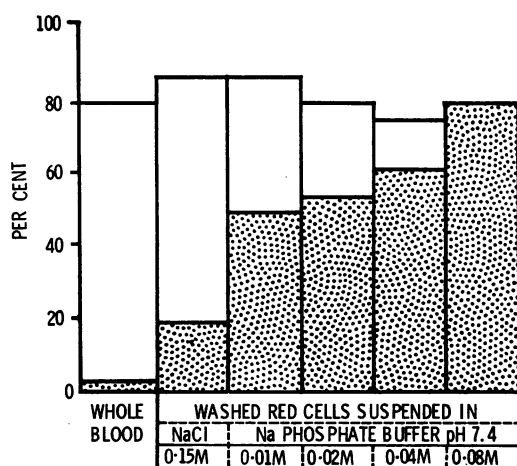


FIGURE 8 Comparison of conversion of pyridoxine (500 ng/ml) to *L. casei* active forms of B<sub>6</sub> after incubation for 60 min with (a) whole blood, and (b) washed red cells suspended in either 0.15 M NaCl or increasing concentrations of phosphate buffer in 0.15 M NaCl. (Shown on abscissa as *L. casei* active B<sub>6</sub> as per cent of added pyridoxine). □ Pyridoxal phosphate + pyridoxal; shaded area represents pyridoxal phosphate.

pyridoxal phosphate was 3% in whole blood, 19% in the saline suspension, and increased progressively in the phosphate buffer suspensions to 100% at 0.08 M.

500 ng of pyridoxine-<sup>3</sup>H per ml was incubated for varying times with (a) whole blood (b) twice washed red cells in 0.15 M NaCl, either intact or hemolyzed in each case. Cells were hemolyzed by ultrasonic disintegration for 3 min. The conversion to pyridoxal phosphate plus pyridoxal and in addition the uptake of pyridoxine by intact cells were compared (Fig. 9), and both were approximately the same in saline suspension as in whole blood.

In hemolyzed cells conversion was lower than in intact cells, being markedly lower in hemolyzed whole blood. In the latter, the form was entirely pyridoxal phosphate, and there was a large proportion in the hemolyzed washed cells.

#### INCUBATION OF DIFFERENT FORMS OF B<sub>6</sub> WITH BLOOD

**Pyridoxamine.** The actual uptake of pyridoxamine into the cells could not be studied by our methods. When 500 ng was incubated per ml of blood for varying times, an increase in pyridoxal phosphate and pyridoxal was measured in the red cells, but only 50% had been converted at 2 hr.

**Pyridoxal.** Amounts of 50–2000 ng of pyridoxal were incubated per ml of blood for 60 min. The form was unchanged, and the distribution between plasma and red cells was approximately the same (mean 32.8% in plasma, 62.5% in cells) (Fig. 10), being also similar to that of pyridoxal converted from pyridoxine by red

cells (Fig. 5). Further, when 500 ng of pyridoxal was incubated with blood for 1 min, there was equilibration to the same distribution which remained the same up to the 2 hr studied (mean 31.5% in plasma, 67.4% in cells).

On the other hand, when varying amounts of pyridoxal were incubated with washed cells in 0.15 M NaCl most of the pyridoxal entered the cells within 1 min, leaving only a trace in the saline.

**Pyridoxal phosphate.** Amounts of 200–2000 ng of pyridoxal phosphate (in terms of pyridoxal) were incubated per ml of (a) whole blood and (b) washed cells in 0.15 M NaCl for 60 min. In whole blood pyridoxal phosphate remained in plasma with no significant amounts entering the red cells. In contrast, when pyridoxal phosphate was incubated with washed cells in 0.15 M NaCl, pyridoxal phosphate entered the washed red cells, only 20% remaining in the plasma even when 2000 ng was added.

**Interconversion of pyridoxal and pyridoxal phosphate.** When either pyridoxal or pyridoxal phosphate was incubated with whole blood, the form remained unchanged. When either form was incubated with washed cells suspended in phosphate buffer (0.08 M) almost half the added pyridoxal was phosphorylated to form pyridoxal phosphate, but pyridoxal phosphate, though entering the cells, did not change. However, if pyridoxal phosphate was incubated with washed cells suspended in 0.15 M NaCl, at least half was dephosphorylated to form pyridoxal.

**Pyridoxine phosphate.** Pyridoxine phosphate was incubated with (a) whole blood and (b) washed cells suspended in 0.15 M NaCl (500 ng/ml in terms of pyri-

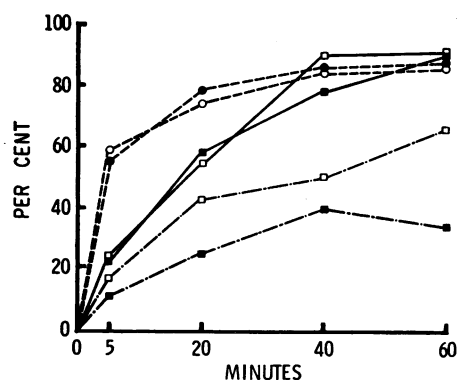


FIGURE 9 Comparison of red cell uptake of pyridoxine-<sup>3</sup>H (500 ng/ml) incubated for different lengths of time with (a) whole blood and (b) washed red cells in 0.15 M NaCl, and comparison of subsequent conversion to *L. casei* active B<sub>6</sub> (pyridoxal phosphate plus pyridoxal) in intact and hemolyzed red cells. (Shown on abscissa as B<sub>6</sub> as per cent of added pyridoxine). Whole blood, ■; red cells, ●; washed red cell suspension, □; washed red cells, ○; —, intact cells; — · — · —, hemolyzed cells; ---, total red cell uptake of pyridoxine.

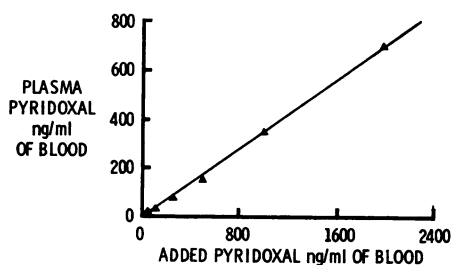


FIGURE 10 Relationship of amount of pyridoxal remaining in plasma with the amount of added pyridoxal incubated with blood.

doxine). In contrast to pyridoxal phosphate, pyridoxine phosphate appeared to enter red cells whether washed or in whole blood, for in both cases approximately 60% was converted in 60 min to pyridoxal phosphate plus pyridoxal in a similar proportion to that found after conversion of pyridoxine.

## DISCUSSION

An increase in pyridoxal phosphate in red cells after treatment of two patients with pyridoxine for 1 wk was observed by Hamfelt (8). As this occurred within a week, he deduced that red cells in circulation played a part in the formation of pyridoxal phosphate. Hamfelt (8) and Yamada and Tsuji (9) observed a rapid passage of  $B_6$  into red cells and some increase in the pyridoxal phosphate concentration, but their studies were confined to the incubation of these compounds with suspensions of washed red cells in phosphate-glucose solution.

In the work described in this paper we were primarily concerned with *in vitro* studies in whole blood (a physiological milieu), which were carried out as a result of our initial observation that active forms of  $B_6$  appeared in red cells immediately after oral administration of pyridoxine. These studies confirmed that pyridoxine was taken up by the red cells, where it was converted to pyridoxal phosphate and then to pyridoxal, followed by a gradual release of a proportion of pyridoxal into the plasma (Figs. 1 and 2). This suggested that pyridoxine may be converted to pyridoxal phosphate in red cells by the pathway demonstrated in liver homogenates (5), and finally to pyridoxal before release into plasma (Fig. 11). It was also claimed that pyridoxine could be converted directly to pyridoxal by an alternative but much slower pathway in the liver (5). The possibility of a small contribution via this pathway in the red cells cannot be excluded.

We have studied, where it is possible, the intermediate stages in the conversion (Fig. 11). Although the first stage, the formation of pyridoxine phosphate, could not be measured directly by the methods used in this work,

the presence of the phosphorylating enzyme, pyridoxal kinase, already shown to be present in red cells (8), was demonstrated by the fact that pyridoxal phosphate formed when pyridoxal was incubated with washed red cells suspended in phosphate buffer. The second stage was demonstrated by the fact that pyridoxine phosphate was converted in whole blood to pyridoxal phosphate, and the final stage by showing that a significant proportion of pyridoxal phosphate incubated with washed cells in saline was dephosphorylated to pyridoxal, presumably demonstrating the presence of the appropriate phosphatase.

The inactivation of this phosphatase was demonstrated when pyridoxine was incubated with stored blood or with red cells suspended in phosphate buffer (Figs. 7 and 8). Under these conditions, though pyridoxal phosphate formed as usual, there was a marked decrease in the conversion of pyridoxal phosphate to pyridoxal. Inorganic phosphate forms in the red cells of stored blood following the breakdown of organic phosphates (10). As the action of phosphatases is known to be minimized in the presence of excess phosphate (11), this could be the explanation for the inactivation of the enzyme in both instances.

Although no significant amounts of pyridoxal phosphate could be detected in plasma at any stage of incubation of pyridoxine with blood, or up to 3 hr after oral ingestion, small amounts of pyridoxal phosphate such as are found normally in plasma (less than 20 ng/ml) might not be detected with certainty, partly because pyridoxal phosphate is estimated indirectly in our method and partly because most of the assays in these experiments were carried out at high dilutions. Very small increases in the serum pyridoxal phosphate concentrations have, in fact, been demonstrated within an hour of intravenous injection of pyridoxine (12, 13). Pyridoxal phosphate was measured directly by enzymatic methods. Also, a small increase in the concentration of pyridoxal phosphate in serum was measured

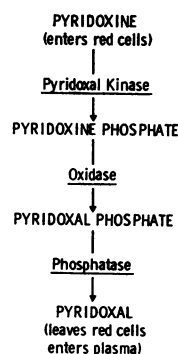


FIGURE 11 Suggested pathway of conversion of pyridoxine to pyridoxal in blood.

after oral ingestion of 2 or 25 mg reaching a small peak at 3 hr (13), unlike the large peak of pyridoxal measured by us much earlier, i.e. about 1 hr after ingestion (Fig. 1).

The distribution of pyridoxal between plasma and red cells was constant during incubation after red cell conversion of pyridoxine (Fig. 5) and after addition of pyridoxal to blood (Fig. 10), irrespective of the amounts added. The fact that the proportion of pyridoxal in plasma was also dependent on the amount of plasma present (Fig. 6) suggests that a factor in plasma, perhaps a specific protein, controls this. If pyridoxal is bound to a protein, the binding is loose, for *L. casei* is able to utilize all the pyridoxal after incubation of blood with pyridoxine or pyridoxal without previous extraction.

The instant equilibration of pyridoxal between plasma and red cells was in contrast to the slower pattern of uptake of pyridoxine (Fig. 2), which is probably partly controlled by the comparative delay in conversion to active forms. The uptake of pyridoxine was unchanged in washed cells in saline (Fig. 9), suggesting a controlling receptor inside the cell for pyridoxine. On the other hand, almost all pyridoxal instantly entered the cells under these conditions, which corroborates the finding that converted pyridoxal inside the cell is not released in the absence of plasma. Pyridoxal phosphate added to whole blood remained in the plasma and did not enter the cells, although it readily entered washed cells in saline. This suggested that a firm binding to a plasma protein might prevent its entry into cells, which was not the case with pyridoxal or pyridoxine. The contrasting behavior of the different B<sub>6</sub> forms is marked. There is evidence that they vary in their affinity for protein, and this has initiated investigations into the protein binding of B<sub>6</sub>.

Not only was pyridoxal phosphate not able to enter the red cell, but it appears that if it formed intracellularly and persisted, as demonstrated in stored blood, it did not leave the cell (Fig. 7). But there is also evidence that in stored blood the factor controlling pyridoxal levels in plasma deteriorates, for when pyridoxal was incubated with stored blood, although pyridoxal phosphate did not form, the distribution was changed with less pyridoxal remaining in the plasma. This could not, however, explain the sharp decrease of pyridoxal found in plasma when pyridoxine was incubated with stored blood, and most probably the explanation is the inability of pyridoxal phosphate to leave the cell; hence its conversion to pyridoxal under normal circumstances for release into plasma.

The findings of this work suggest that all forms of B<sub>6</sub> incubated with and entering the red cells are converted mainly via pyridoxal phosphate ultimately to pyridoxal, a proportion of which is then gradually re-

leased into plasma. It is not known whether this conversion affects the red cell function but it is interesting that pyridoxal phosphate, like 2,3-diphosphoglycerate and ATP causes a shift to the right in the oxygen dissociation curve (14-16).

It is also not certain whether pyridoxal released into plasma is utilized elsewhere. It is possible that pyridoxal is the main transport form and that following its easy passage into cells of other tissues of the body, for example marrow cells, pyridoxal phosphate, the coenzyme, forms inside the cell. This could explain the significance of the wide distribution of pyridoxal kinase in different tissues (17). However, pyridoxal phosphate could only form and remain as such provided that there was not a phosphatase also present capable of breaking down pyridoxal phosphate. Alternatively, this pyridoxal might be mainly excreted after conversion to pyridoxic acid in the liver.

Homogenates of liver and brain have been shown to convert pyridoxine either to pyridoxal phosphate or to pyridoxal (5). It should be taken into account that it is likely that these homogenates contained some red cells, and we have shown that a very small number of red cells can convert a significant amount of pyridoxine (Fig. 3). We have carried out preliminary studies with white blood cells and marrow cells but have not been able to demonstrate that these cells can convert any significant amounts of pyridoxine.

It is interesting that where other vitamins are concerned, there have been various reports of uptake into red cells and the consequent appearance of related forms in the cells (18-23). However, it is not known whether the red cell converts these other vitamins to active forms which are also subsequently released into plasma.

Investigations are in progress into the conversion of pyridoxine to active forms in blood in various conditions. A condition of particular interest is the alcohol-induced sideroblastic anemia. Recently, evidence of an impaired conversion of pyridoxine to pyridoxal phosphate in the body has been demonstrated in this condition (24). If the conversion in blood is of importance in the body, the demonstration of a defect at any stage might have some bearing on this, and on the pyridoxine-responsive sideroblastic anemias.

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