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PYRIDOXINE (PYRIDOXAMINE) PHOSPHATE OXIDASE ACTIVITY IN THE RED CELL

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Summary

A method has been developed for the measurement of red cell pyridoxine (pyridoxamine) phosphate oxidase activity. The more stable substrate, pyridoxamine phosphate, was incubated with a red cell haemolysate and the product pyridoxal phosphate was measured by *Lactobacillus casei* microbiological assay.

A wide range of oxidase activities was found in control subjects (6—136 ng pyridoxal phosphate/g Hb $\times 10^{-2}$). There is a close correlation between the rate of conversion of pyridoxine to pyridoxal phosphate in whole blood and the oxidase activity. There was a marked increase in both after oral riboflavin. These results suggest that the oxidase plays a large part in the regulation of vitamin B-6 metabolism in red cells.

Introduction

The mature red cell is an active site of metabolism of vitamin B-6 [1,2]. Pyridoxine and pyridoxamine are taken up by red cells where they are phosphorylated by a kinase, followed by oxidation to pyridoxal phosphate by an oxidase and hydrolysis by a phosphatase to pyridoxal, the form that is released into plasma. The rate of red cell conversion of pyridoxine to pyridoxal phosphate has been measured in whole blood by Anderson et al. [1,3,4].

Activity of the kinase (ATP:pyridoxal 5'phosphotransferase, EC 2.7.1.35) has been studied extensively in the red cell [5—9] but, in spite of the major role that the oxidase plays in the formation of pyridoxal phosphate, there are

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only two reports of measurement of activity of this enzyme in red cells [2,10]. Pyridoxine (pyridoxamine) phosphate (pyridoxamine-phosphate:oxygen oxidoreductase (deaminating), EC 1.4.3.5) which was first studied in rabbit liver [11—13] catalyses the conversion of both pyridoxine phosphate and pyridoxamine phosphate to pyridoxal phosphate [14,15]. The prosthetic group of the enzyme is FMN, a phosphorylated derivative of riboflavin, and the reaction requires oxygen [13—16].

Thus the reactions are as follows:

$$\text{Pyridoxine phosphate} + O_2 \xrightarrow{\text{FMN}} \text{pyridoxal phosphate} + H_2O_2$$

$$\text{Pyridoxamine phosphate} + O_2 + H_2O \xrightarrow{\text{FMN}} \text{pyridoxal phosphate} + H_2O_2 + NH_3$$

Morisue and coworkers [13] studied the oxidase activity in various animal tissues and reported that it is confined to a few tissues, including red cells. The highest activity was found in liver and activity in red cells was of a similar order to that in the other tissues, but no activity was found in bone marrow. Lumeng and Li [2] located the red cell oxidase in the supernatant fraction of a haemolysate.

This paper reports investigations into a new method for the measurement of B-6 oxidase activity in red cells. Red cell oxidase activity is compared with the rate of conversion of pyridoxine to pyridoxal phosphate in whole blood in control subjects.

Materials and Methods

Materials. Vitamin B-6 compounds were obtained from Sigma Chemical Co. Ltd. All other reagents were obtained from BDH Ltd.

Methods. Venous blood was collected with heparin as anticoagulant. After centrifugation at 1500 × *g*, plasma and white cells were removed and the red cells washed three times with 0.154 M NaCl at 4°C. The upper layer of red cells (approx. 20%), containing reticulocytes, was removed and a dilution of 1 in 4 with distilled water was made from the remaining cells. The haemoglobin concentration was measured in this haemolysate which was further diluted to give a final dilution of red cells in distilled water of 1 in 10 (final haemoglobin concentration approx. 2.5 g/100 ml). The haemolysate was frozen and thawed to ensure complete haemolysis and centrifuged at 1800 × *g* to remove red cell ghosts. 0.1 ml of 0.8 M sodium phosphate buffer (pH 8.0) was added per ml of supernatant fraction in order to inhibit the phosphatase [1,2], and this was incubated at 37°C for 15 min before addition of substrate. An amount of pyridoxamine phosphate equivalent to 2 μg pyridoxamine was added per ml reaction mixture. Two aliquots were incubated at 37°C, removed at 1 and 2 h, respectively, and immediately frozen at —25°C to prevent further oxidation of pyridoxamine phosphate to pyridoxal phosphate. Pyridoxal phosphate after acid hydrolysis to pyridoxal was measured in these samples and in the reaction mixture before addition of pyridoxamine phosphate, using *Lactobacillus casei* NCIB 8010 (ATCC 7469) microbiological assay [17]. This assay measures only pyridoxal and therefore does not measure pyridoxamine compounds. Care was taken to protect samples from light since vitamin B-6 compounds are light-

sensitive. The oxidase activity is the increase in pyridoxal phosphate at 2 h expressed in terms of pyridoxal as ng/g Hb $\times 10^{-2}$.

The rate of conversion of pyridoxine to pyridoxal phosphate in whole blood was measured as described previously [1,4] and expressed as ng pyridoxine converted at 20 min/g Hb $\times 10^{-2}$.

Subjects studied. 35 healthy subjects, members of staff, mainly of Anglo-Saxon origin, with no evidence of riboflavin or vitamin B-6 deficiency and aged 19—57 years were studied. 5 were known from previous studies to have slow red-cell conversion rates of pyridoxine to pyridoxal phosphate.

Results

Substrate concentration

Formation of pyridoxal phosphate increased with increasing substrate concentration in all three haemolysates (Fig. 1).

The V for the reaction was 25 nmol pyridoxal phosphate/g Hb per h and the apparent K_m was 1.3 μM. An amount of pyridoxamine phosphate equivalent to 2 μg pyridoxamine/ml haemolysate was chosen as a suitable concentration.

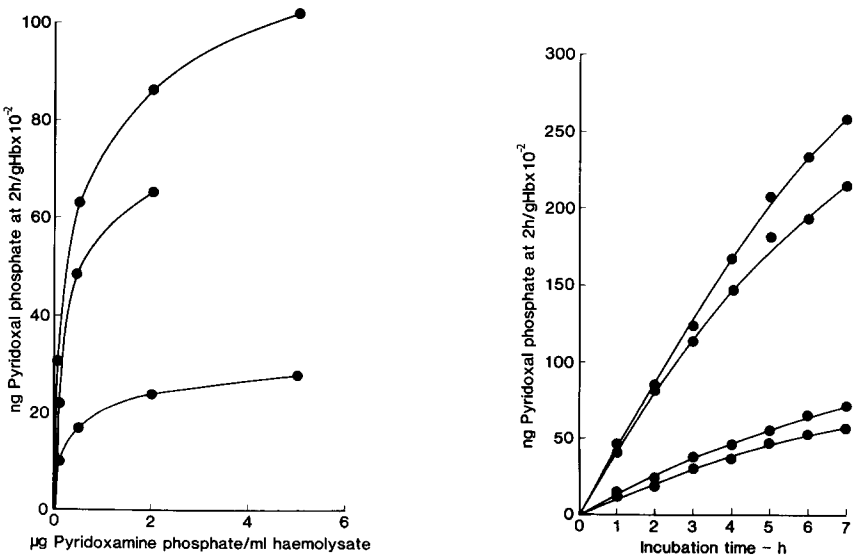


Fig. 1. Effect of substrate (pyridoxamine phosphate) concentration on the formation of pyridoxal phosphate. Different concentrations of pyridoxamine phosphate (equivalent to 0.1, 0.5, 2 and 5 μg pyridoxamine/ml haemolysate) were incubated for 2 h with haemolysates from 3 subjects with widely different oxidase activities. Pyridoxal phosphate was expressed in terms of pyridoxal.

Fig. 2. Effect of incubation time on the formation of pyridoxal phosphate from pyridoxamine phosphate. Haemolysates from 4 subjects with varying oxidase activity were incubated with pyridoxamine phosphate (equivalent to 2 μg pyridoxamine/ml haemolysate) for periods up to 7 h. Pyridoxal phosphate was expressed in terms of pyridoxal.

Rate of product formation

Pyridoxal phosphate formation increased with time in all the haemolysates studied (Fig. 2). The increase was linear for a longer period the higher the activity, i.e., for 3 h with the lowest activity and for 5 h with the highest activity. An incubation time of 2 h was chosen because there was better differentiation between low and high activities at this time than at 1 h.

Validity of method as a measurement of oxidase activity

The formation of pyridoxal phosphate when pyridoxamine phosphate is incubated with a haemolysate would be expected to be the result of a single oxidation step catalysed by the oxidase. However, if pyridoxamine phosphate was unstable like pyridoxine phosphate, it could first break down to pyridoxamine and the formation of pyridoxal phosphate would result from a two-step process involving both the kinase and the oxidase. The following experiments were carried out to confirm that the method is a measure of the oxidase activity alone.

1. Effect of Mg^{2+} and 4-deoxypyridoxine. The conversion of pyridoxine and pyridoxamine to pyridoxal phosphate, in which the Mg^{2+}-dependent kinase is involved, is approximately doubled by the addition of Mg^{2+} and halved in the presence of the antagonist, 4-deoxypyridoxine, said to be an inhibitor of the kinase [18,19]. In contrast, the conversion of pyridoxamine phosphate to pyridoxal phosphate was unaffected by either of these additions (Table I).

2. Incubation of B-6 compounds in suspensions of red cells in plasma, washed red cells in 0.154 M NaCl, and in a haemolysate. Conversion of both pyridoxine and pyridoxamine to pyridoxal phosphate occurred at a much faster rate in a suspension of red cells in plasma and washed red cells in 0.154 M NaCl than in a haemolysate, probably because ATP which is required for kinase activity is lost during haemolysis (Table II). In striking contrast, no pyridoxal phosphate was formed from pyridoxamine phosphate incubated with whole cells. However, when pyridoxamine phosphate was in direct contact with the oxidase in a haemolysate, there was a significant conversion to pyridoxal phosphate, greater than from pyridoxamine.

Effect of pH

Haemolysates were prepared by dilution of red cells in 80 mM sodium phos-

TABLE I

EFFECT OF Mg^{2+} AND 4-DEOXYPYRIDOXINE ON THE FORMATION OF PYRIDOXAL PHOSPHATE FROM DIFFERENT B-6 COMPOUNDS

2 μg pyridoxine, 2 μg pyridoxamine or 2.95 μg pyridoxamine phosphate (equivalent to 2 μg pyridoxamine) were each incubated per ml reaction mixture with (1) no additions; (2) 1 mM $MgCl_2$; (3) 0.2 mM 4-deoxypyridoxine. Pyridoxal phosphate was expressed in terms of pyridoxal.

Substrate	ng pyridoxal phosphate at 2 h/g Hb $\times 10^{-2}$		
	(1)	(2)	(3)
Pyridoxine	59	109	30
Pyridoxamine	21	44	10
Pyridoxamine phosphate	89	92	90

TABLE II

FORMATION OF PYRIDOXAL PHOSPHATE FROM DIFFERENT B-6 COMPOUNDS IN SUSPENSIONS OF RED CELLS IN PLASMA, WASHED RED CELLS IN 0.154 M NaCl, AND IN A HAEMOLYSATE

2 μg pyridoxine, 2 μg pyridoxamine or 2.95 μg pyridoxamine phosphate (equivalent to 2 μg pyridoxamine) per ml were each incubated at 37°C for 2 h in shaking water bath in the presence of 80 mM sodium phosphate buffer (pH 8.0) with (1) red cells suspended in plasma; (2) washed red cells suspended in 0.154 M NaCl; (3) a red cell haemolysate. The dilution of red cells was 1 in 10 and the haemoglobin concentration in each was approx. 2.5 g/100 ml. Pyridoxal phosphate was expressed in terms of pyridoxal.

Substrate	ng pyridoxal phosphate at 2 h/g Hb × 10^{-2}		
	(1)	(2)	(3)
Pyridoxine	159	109	40
Pyridoxamine	57	53	6
Pyridoxamine phosphate	0	0	46

phate (pH 7.4 and 8.0) and 80 mM sodium carbonate (pH 9.0 and 9.5) instead of distilled water, and oxidase activity was measured. The pH optimum was 8.0.

Effect of haemoglobin concentration in the reaction mixture

Red cells were diluted to give haemolysates of different haemoglobin concentration (1.0—2.8 g/100 ml) and therefore of different enzyme concentration. Oxidase activity (ng pyridoxal phosphate/g Hb × 10^{-2}) was approximately the same over this range of haemoglobin concentrations (mean ± S.D. = 42.8 ± 1.2). However, whenever there were sufficient cells, a haemolysate with a haemoglobin concentration of approx. 2.5 g/100 ml was used in the assay.

Preparation of haemolysate

The oxidase activity was approximately the same in a subject with a normal reticulocyte count whether the uppermost layer of red cells containing reticulocytes, was removed or not (Table III, (1) and (2)). However, the activity

TABLE III

METHOD OF PREPARATION OF HAEMOLYSATE

Oxidase activity in subjects with normal reticulocyte counts was measured in red-cell haemolysates (1 in 10 dilution) prepared as follows: From packed red cells prepared as described in Materials and Methods from which (1) the white cell layer had been removed; (2) the white cell layer and approx. 20% of the red cells at the top had been removed. From the top half (3a) and the botton half (3b) of packed red cells in 0.154 M NaCl after centrifugation at 1500 × *g* for 30 min and removal of white cell layer. Pyridoxal phosphate was expressed in terms of pyridoxal.

Subjects	Oxidase activity (ng pyridoxal phosphate at 2 h/g Hb × 10^{-2})			
	(1)	(2)	(3a)	(3b)
1	86	82	—	—
2	83	82	100	66
3	81	79	98	69
4	—	—	79	57
5	—	—	22	12

was much greater in red cells from the top half, presumably containing reticulocytes and younger cells, than in red cells from the bottom half, containing older cells (Table III, (3a) and (3b)). As this suggests that younger cells have a higher activity we chose to use method (2) in the assay system so that oxidase activity in control subjects and patients with raised reticulocytes counts could be more accurately compared. Further studies to measure oxidase activity in red cells at all stages of their development are now being carried out.

Effect of storage of haemolysates

13 haemolysates from subjects with varying oxidase activity were stored at −25° C for periods of up to 2 weeks. Oxidase activity remained approximately the same during this period (coefficient of variation 4.2%).

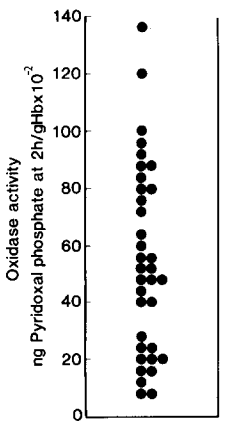
Reproducibility

Oxidase activity was measured in a normal subject 11 times over a period of 15 months. The mean ± S.D. was 85.8 ± 8.0 ng pyridoxal phosphate at 2 h/g Hb $\times 10^{-2}$. One value was markedly higher than the rest for an unknown reason and when excluded, the mean ± S.D. was 83.8 ± 4.6 ng pyridoxal phosphate. In another 12 subjects with widely varying oxidase activity, which was measured on two occasions (2 weeks to 11 months apart), the coefficient of variation was 21.4%. As this would reflect any variation in an individual, in the test and the B-6 assay, this demonstrates that the method is reproducible and that the oxidase activity is fairly constant.

Oxidase activity in normal subjects

There was a wide variation in the oxidase activity (6—136 ng pyridoxal phosphate at 2 h/g Hb $\times 10^{-2}$) in 35 healthy subjects (Fig. 3).

Fig. 4 demonstrates that there is a close correlation of the oxidase activity



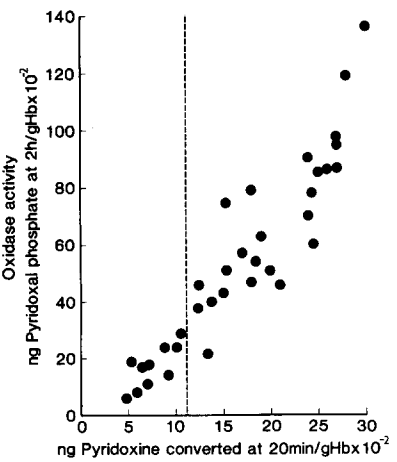


Fig. 3. Red-cell pyridoxine phosphate oxidase activity in 35 control subjects. Pyridoxal phosphate was expressed in terms of pyridoxal.

Fig. 4. Correlation of red-cell pyridoxine phosphate oxidase activity with the rate of pyridoxine conversion to pyridoxal phosphate in whole blood in 35 control subjects. Pyridoxal phosphate was expressed in terms of pyridoxal. The broken line represents the arbitrary cut-off point in control subjects below which subjects are referred to as 'slow converters' [4].

TABLE IV

EFFECT OF FMN IN VITRO ON PYRIDOXINE PHOSPHATE OXIDASE ACTIVITY

Oxidase activity was measured alone and after incubation for 30 min with FMN (0.1 and 0.3 μg/ml haemolysate) in haemolysates from 3 subjects with varying oxidase activity. Pyridoxal phosphate was expressed in terms of pyridoxal.

Subject	Oxidase activity (ng pyridoxal phosphate at 2 h/g Hb × 10^{-2})		
	No addition	0.1 μg FMN/ml	0.3 μg FMN/ml
1	80	126	128
2	12.5	24	29
3	9	24	28

TABLE V

EFFECT OF ORAL RIBOFLAVIN ON RED CELL PYRIDOXINE PHOSPHATE OXIDASE ACTIVITY AND ON THE RATE OF CONVERSION OF PYRIDOXINE TO PYRIDOXAL PHOSPHATE IN WHOLE BLOOD

4 subjects with low red-cell oxidase activity and slow rate of conversion of pyridoxine to pyridoxal phosphate in whole blood were given oral riboflavin (24 mg/day for 2 weeks to subjects 1 and 2; and 30 mg/day for 5 weeks to subject 3 and 4). Pyridoxal phosphate was expressed in terms of pyridoxal.

Subjects	Oxidase activity (ng pyridoxal phosphate at 2 h/g Hb × 10^{-2})		Pyridoxine conversion rate (ng pyridoxine converted at 20 min/g Hb × 10^{-2})	
	Before riboflavin	After riboflavin	Before riboflavin	After riboflavin
1	11	26	7	15
2	6	25	5	12
3	5	27	4	14
4	5	23	5	16

with the rate of conversion of pyridoxine to pyridoxal phosphate in whole blood ($r = 0.944; P < 0.001$).

Effect of riboflavin

In vitro addition of FMN resulted in a marked increase in oxidase activity in three subjects, whether the initial activity was low or high (Table IV).

After oral riboflavin given to four subjects who had a low initial oxidase activity, there was also a marked increase in activity and a corresponding increase in the rate of conversion of pyridoxine to pyridoxal phosphate in whole blood (Table V).

Discussion

We reported in a previous study a wide variation in the rate of red cell metabolism of vitamin B-6 in apparently healthy individuals as measured by the rate of conversion of pyridoxine to pyridoxal phosphate in whole blood [20,3,4]. It was demonstrated that this is a familial characteristic of an

inherited nature [21]. Stimulation of the rate of conversion by oral riboflavin, particularly marked when the rate was slow [22,4], suggested that the FMN-dependent oxidase played a large part in regulating the red cell metabolism of B-6.

In order to measure the activity of this enzyme the obvious choice of substrate was pyridoxine phosphate, which had been used to demonstrate the presence of the oxidase in the red cell [1], and was the substrate used in one of the reported studies of red cell B-6 oxidase [2].

However, we found that its unstable nature made it impossible to develop a reliable assay using pyridoxine phosphate as substrate. As the maximum velocity of the enzyme under optimal conditions is the same for pyridoxamine phosphate [14,15], and this compound is stable and easily available, we used pyridoxamine phosphate (as did Lakshmi and Bamji [10]), in the development of an assay for the B-6 oxidase. In this paper, optimal conditions have been established for a sensitive method. The stability of the substrate in the assay system was demonstrated (Tables I and II) and it was established that the method is a measure of oxidase activity alone. It is of particular interest that 4-deoxypyridoxine has no effect on the oxidase activity (Table I), also shown by Wada and Snell [14], but inhibits the kinase as previously suggested [18,19].

A wide variation was found in the red cell oxidase activity in control subjects with no evidence of riboflavin deficiency (Fig. 3). The close correlation with the rate of conversion of pyridoxine to pyridoxal phosphate in whole blood ($P < 0.001$) (Fig. 4) confirmed the suggestion that the oxidation is usually the rate limiting step in this conversion. In the study of the rate of conversion of pyridoxine to pyridoxal phosphate in whole blood in 79 control subjects [4], we chose an arbitrary cut-off point below which 5 were said to be 'slow converters' because their rate of conversion was much slower than that of the remaining subjects. The 11 subjects with markedly low oxidase activities (Fig. 3) were all 'slow converters', with the exception of one whose rate of conversion was just above the cut-off point.

There was a marked increase in the oxidase activity in slow converters after a short course of oral riboflavin (Table V) which corresponded to an increase in the rate of conversion of pyridoxine to pyridoxal phosphate in whole blood. There were similar increases when patients with riboflavin deficiency were given oral riboflavin by Lakshmi and Bamji [10]. The stimulation can be explained by an increase in FMN formed in the red cell from riboflavin [23—25]. The finding that the oxidase activity increased after in vitro addition of FMN even in a subject with a normal rate of conversion (Table IV) confirmed earlier observations that oral riboflavin increased the rate of B-6 metabolism in the red cell in subjects with normal rates [4]. This suggests that the oxidase is not normally maximally active.

Further studies suggest that the wide range of oxidase activities, which is reported here, is due to variation in the rate of metabolism of riboflavin in the red cell (unpublished data).

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