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High-performance liquid chromatographic assay of human lymphocyte kynureninase activity levels

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ABSTRACT

Human lymphocyte kynureninase activity was assessed in homogenized cells by determination of 3-hydroxyanthranilic acid production as a function of time after addition of the substrate, 3-hydroxykynurenine. The product, 3-hydroxyanthranilic acid, was determined by isocratic high-performance liquid chromatography and fluorescence detection. Mean (± S.D.) lymphocyte kynureninase activity in a group ($n = 12$) of vitamin B_6-deficient men was 5.04 ± 0.81 pmol 3-hydroxyanthranilic acid formed per mg protein per min, which was significantly ($p = 0.005$) lower than the 6.69 ± 1.70 pmol 3-hydroxyanthranilic acid formed per mg protein per min in men with a normal vitamin B_6 status. This indicates that lymphocyte kynureninase activity is depressed during a vitamin B_6 deficiency.

INTRODUCTION

In the oxidative metabolism of tryptophan the enzyme kynureninase (EC 3.7.1.3) catalyzes the conversion of L-3-hydroxykynurenine and L-kynurenine to 3-hydroxyanthranilic acid (3-HAA) and anthranilic acid, respectively [1]. The reaction is pyridoxal-5′-phosphate (PLP)-dependent and has been reported to be sensitive to nutritional vitamin B_6 deprivation in mammals [2,3]. An experimentally induced vitamin B_6 deficiency is characterized by accummulation of kynurenine, 3-hydroxykynurenine, kynurenic and xanthurenic acids, especially after an oral tryptophan loading dose [4]. Determination of urinary excretion of the above-mentioned compounds before and after a tryptophan loading dose is often used as a functional index of vitamin B_6 nutritional status [5]. However, several factors unrelated to vitamin B_6 nutritional status (*i.e.* glucocorticoid hormones, viral infections, oral contraceptives) also affect tryptophan metabolism, resulting in an urinary excretion pattern of tryptophan metabolites indicative of a vitamin B_6 deficiency [6].

The use of oral contraceptives serves as an example where abnormal tryptophan metabolism has incorrectly conveyed the impression of a vitamin B_6 deficiency. Based on the tryptophan load test, women receiving oral contraceptives have been believed to be vitamin B_6-deficient [7,8]; however, Bender and Wynick

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[9] demonstrated that these results merely reflected the inhibition of kynureninase by estrogen. The pitfalls of the tryptophan load test prompted us to investigate whether direct determination of kynureninase activity levels is more feasible to study the relationship between vitamin B_6 status and tryptophan metabolism.

The enzyme kynureninase is well expressed in mammalian livers [10], but activity of this enzyme in spleen, lung, brain, heart and muscle is much lower [10,11]. It is not known if the enzyme is also expressed in blood cells. We therefore investigated (a) whether the enzyme is expressed in human blood cells, and, if present, (b) whether the enzyme assay would reflect vitamin B_6 nutritional status.

EXPERIMENTAL

Materials

All reagents were obtained from Merck (Darmstadt, Germany), except for 3-hydroxykynurenine and 3-HAA, which were purchased from Sigma (St. Louis, MO, U.S.A.).

Assay for kynureninase activity

Lymphocytes were isolated from human blood collected with EDTA as anticoagulant. To 10 μl of packed lymphocytes, 225 μl of ice cold sodium 5,5-diethylbarbiturate–HCl buffer (pH 8.4) and 25 μl of a 10% Triton X 100 solution were added. After sonication, 100 μl of the cell homogenate were added to 20 μl of a PLP solution (24 μM), or to 20 μl of barbiturate buffer. Cell homogenates were then preincubated at either 15 or 30°C for 10 min; the reaction was subsequently started by addition of 3-hydroxykynurenine to a final concentration of 1 mmol/l. Except for linearity studies, where longer incubation times were used, the reaction was terminated after 5 min by addition of 150 μl of 10% trichloroacetic acid. The clear supernatant obtained after centrifugation was used for HPLC analysis of 3-HAA. An aliquot of the cell homogenate was used to determine the protein content (Bio-Rad protein assay kit; Bio-Rad Labs., Munich, Germany), and enzyme activity was expressed as pmol 3-HAA formed per milligram protein per minute.

High-performance liquid chromatography (HPLC)

A Perkin-Elmer (Norwalk, CT, U.S.A.) Series II pump was fitted with a Whatman (Clifton, NJ, U.S.A.) Partisphere C_{18} column (150 mm × 4.6 mm I.D.; 5 μm particle size). The analytical column was protected by installation of a Whatman Solvecon pre-column (250 mm × 4.6 mm I.D.) between the pump and the injector and a Whatman reversed-phase guard column between the injector and the analytical column. A Perkin-Elmer LS 4 fluorescence spectrometer was used for detection (excitation wavelength 322 nm; emission wavelength 414 nm) of 3-HAA. The spectral slit widths were chosen to give a bandpass of 10 nm. The HPLC procedure was fully automated by employing a Spectra-Physics (San Jose,

CA, U.S.A.) SP 8780 XR autosampler, coupled to a Spectra-Physics 4270 integrator. A 0.1 *M* KH_2PO_4 buffer (pH 5.8) containing 1% acetonitrile, was used as mobile phase at a flow-rate of 1.0 ml/min. A 30-μl aliquot of the clear supernatant was injected onto the column to determine 3-HAA production by the enzyme-catalyzed reaction.

Relationship with vitamin B_6 status

The vitamin B_6 status of 30 men from a low socio-economic group were determined by assay of plasma PLP levels [12]. Twelve men were definitely vitamin B_6-deficient (PLP $\leq$ 12 nmol/l), while twelve men presented with a low, but still normal, vitamin B_6 status (PLP $>$ 20 nmol/l). These two groups, defined by different plasma PLP levels, were compared with respect to lymphocyte kynureninase activity levels.

RESULTS

Preliminary work in our laboratory indicated that 3-HAA showed optimum fluorescence at pH 5.8. Using therefore a mobile phase (0.1 *M* KH_2PO_4) adjusted to pH 5.8 and containing 1% acetonitrile, 3-HAA was eluted from the column within 3 min. Analysis of a blank lymphocyte homogenate indicated that several

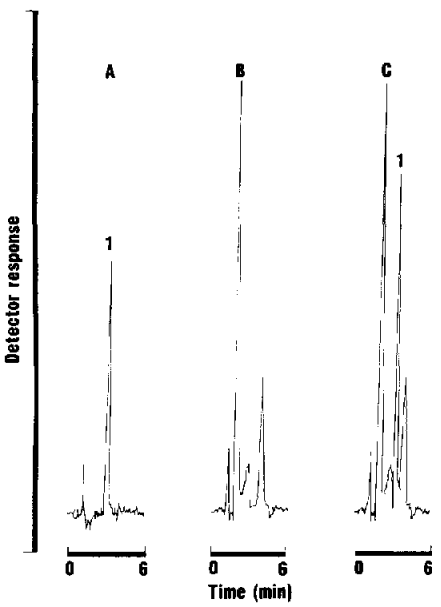

Fig. 1. Determination of lymphocyte kynureninase activity levels using HPLC. Enzyme activity is measured by quantification of product (3-HAA) formation. (A) 3-HAA standard (12.0 nmol/l); (B) lymphocyte homogenate blank; (C) lymphocyte 3-HAA production after 5 min of incubation in presence of 3-hydroxykynurenine. Peaks: 1 = 3-HAA; unmarked peaks are unidentified components.

unidentified components eluted from the column within the first 4 min; however, none of these interfered with quantification of 3-HAA, the product of the reaction catalyzed by kynureninase (Fig. 1). No kynureninase activity was detected in erythrocytes.

When the kynureninase assay was performed at 30°C, linearity could only be achieved in the presence of excess (4 μmol/l) PLP (Fig. 2A). When PLP was absent, the accumulation of 3-HAA was non-linear with respect to incubation time; however, linearity could be restored upon addition of PLP to a final concentration of 4 μmol/l (Fig. 2A). When the enzyme reaction was performed at 15°C, 3-HAA accummulation was linear for up to 10 min in the absence of additional PLP (Fig. 2B).

The enzyme is active over a relative broad pH range, with an optimum recorded at pH 8.4 (Fig. 3). Lymphocyte kynureninase activity levels in vitamin B_6-deficient individuals were significantly ($p = 0.005$; Student's t-test) lower when compared to persons with a normal vitamin B_6 status (Table I).

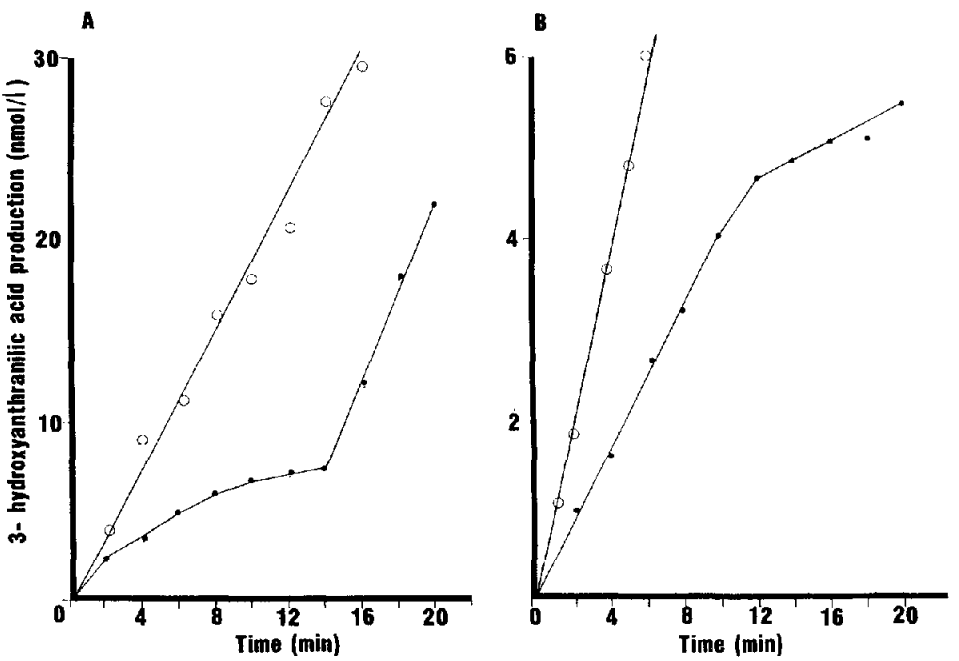

Fig. 2. Human lymphocyte kynureninase activity assayed at 30°C (A) or 15°C (B). For each experiment, 100 μl of packed lymphocytes were used as an enzyme source. After addition of 3-hydroxykynurenine (final concentration: 1 mmol/l), aliquots of 150 μl were removed at 2-min intervals, added to 150 μl of 10% trichloroacetic acid and analyzed for 3-HAA as described under Experimental. Assays were done in presence (○) of added PLP (4 μmol/l), or without additional PLP (●). After 13 min, PLP was also added to the kynureninase assay performed without PLP at 30°C (●, A), thus restoring linearity of the reaction.

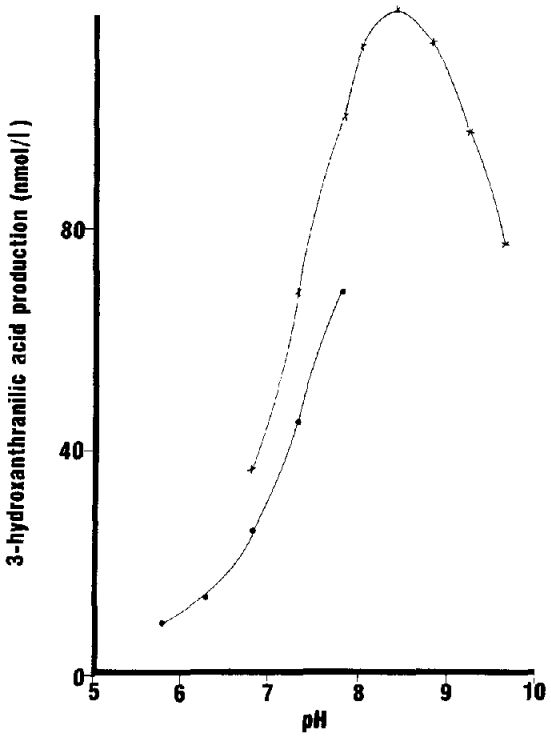

Fig. 3. pH dependency of the kynureninase assay. Assays were carried out in presence of 0.04 *M* sodium phosphate buffer (●) or 0.04 *M* sodium 5,5-diethylbarbiturate–HCl buffer (×).

TABLE I

LYMPHOCYTE KYNURENINASE ACTIVITY LEVELS IN TWO GROUPS OF MEN DEFINED BY VITAMIN B_6 NUTRITIONAL STATE

Group	Group description	Plasma PLP (mean ± S.D.) (nmol/l)	Lymphocyte kynureninase activity[a] (mean ± S.D.)
A	Plasma PLP > 20 nmol/l; normal	26.1 ± 5.1	6.69 ± 1.66
B	Plasma PLP ≤ 12 nmol/l; deficient	9.0 ± 2.0	5.04 ± 0.81[b]

[a] Lymphocyte homogenates were prepared as described under *Experimental*. Kynureninase activity was determined in absence of additional pyridoxal-5'-phosphate (PLP) at 15°C and is expressed as pmol 3-hydroxyanthranilic acid performed per mg protein per min.

[b] Activity levels significantly depressed when compared to group A ($p = 0.005$).

DISCUSSION

Kynureninase and 3-hydroxykynureninase have been considered to be a single enzyme in the mammalian liver [10]. Activity levels of this enzyme have been previously assayed by HPLC in different rat tissues. Kawai *et al.* [11] recently reported an HPLC method to monitor kynureninase-catalyzed anthranilic acid production from L-kynurenine. In our study, we opted for the use of L-3-hydroxykynurenine as substrate for the enzyme, because the enzyme shows a lower Michaelis constant and a higher V_{max} for the latter substrate [13,14]. These favourable kinetic constants, as well as the high fluorescence exhibited by 3-HAA, allowed reproducible assay of kynureninase activity levels from small tissue samples. Due to the high sensitivity of the assay, kynureninase activity levels could be determined in lymphocytes prepared from 10 ml of human blood. For the human liver it has been shown that identical kynureninase enzymes exist in the cytosol and mitochondria [14]; therefore, no effort was made to separate the cytosolic and mitochondrial enzymes in our assay procedure.

The non-linearity of the kynureninase assay when performed at 30°C in absence of PLP (Fig. 2) is strongly reminiscent of the inactivation of this enzyme isolated from *Pseudomonas marginalis* when the reaction was carried out in absence of added PLP [15,16]. For this bacterial enzyme, inactivation was the result of pyridoxamine-5′-phosphate formation from PLP by transamination with L-alanine or ornithine [16]. In presence of pyruvate, inactivation of the bacterial enzyme was prevented [16], indicating that PLP may be regenerated by reverse transamination between pyridoxamine-5′-phosphate and pyruvate. It has not been established whether a similar mechanism could explain the inactivation of the human lymphocyte kynureninase when assayed in absence of PLP. Unlike the bacterial enzyme, addition of pyruvate to the lymphocyte homogenate did not restore kynureninase activity (unpublished observations). Addition of PLP fully restored enzyme activity, indicating that inactivation was only temporary and the result of either inactivation of PLP or dissociation of the holo-enzyme. The latter possibility is favoured by the linearity of the assay, in absence of PLP, during the first 10 min when performed at 15°C (Fig. 2B). In order to study vitamin B_6 nutritional status and tryptophan metabolism, the kynureninase assay has to be performed in absence of additional PLP. To ensure linearity, we now routinely perform this assay at 15°C with an incubation time of 5 min.

Lymphocyte kynureninase activity levels of vitamin B_6-deficient men were significantly lower when compared to men with normal plasma PLP levels (Table I). This difference was obtained although the normal group had a mean plasma PLP level in the lowest quintile of the plasma PLP reference range (reference range: 20–80 nmol/l). This indicates that lymphocyte kynureninase activity levels reflect vitamin B_6 status and may replace the tryptophan load test with subsequent quantification of urinary tryptophan metabolites as a functional index of vitamin B_6 nutritional status.

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