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Effect of vitamin B_6 supplementation on degradation rates of short-lived proteins in human neutrophils

Felicia Stern,* Yitshal N. Berner,† Zeev Polyak,† Margarita Komarnitsky,* Ben-Ami Sela,‡ and Yosef Dror*

**Institute of Biochemistry, Food Sciences and Nutrition, Faculty of Agriculture and Environmental Quality Sciences, The Hebrew University of Jerusalem, Rehovot, Israel; †Subacute Department and Clinical Nutrition, Hartzfeld Geriatric Hospital, Kaplan Medical Center, Gedera, Israel; ‡Institute of Chemical Pathology, Sheba Medical Center, Tel Hashomer, Israel*

Metabolic pathways are controlled primarily by protein degradation rates. Degradation rates, in turn, are controlled by changes in physiologic condition or nutrient supply. Vitamin B_6 is associated with a greater variety of reactions than most other vitamins. Moreover, the vitamin B_6 needs of the elderly tend to be higher than those of young adults. Neutrophils seem to be appropriate cells for assessing protein turnover as affected by macronutrients and micronutrients. Thus, we assumed that vitamin B_6 supplementation, particularly in an elderly population, would change the turnover rates of the neutrophil proteins. Protein synthesis was measured after 30 minutes of ^{35}S-Met incorporation followed by a 30-minute washout incubation; degradation was measured after an additional 5-hour incubation. Following protein separation, radioactive images of short-lived proteins were electronically separated into bands. Vitamin B_6 supplementation significantly increased the synthesis of most neutrophil protein bands. There was a significant decrease of 25 to 66% in the degradation rates of 235 protein bands. We even detected by statistical evaluation a 20% decrease in the degradation rates of distinct protein bands. Activation coefficients of erythrocyte aspartate aminotransferase (AC-AST) decreased markedly. There was a significant positive correlation between the decrease in AC-AST and protein degradation. The N-end rule proposes that pyridoxal 5′-phosphate decreases degradation rates of short-lived proteins by binding to lysyl residues. A biochemical model of the mechanism of cellular protein turnover, as affected by nutritional intervention, in human neutrophils is demonstrated. (J. Nutr. Biochem. 10:467–476, 1999)

Keywords: protein degradation; neutrophil; vitamin B_6; activation coefficient; aspartate aminotransferase; N-end rule

Introduction

Metabolic pathways are controlled primarily by protein degradation rates.[1] A study of the factors controlling these rates is crucial to a better understanding of physiologic status. However, the mechanisms and regulation of degradation for most intracellular proteins remain a biological enigma.[2] One of proteins' main properties is their degradation rate and its dependence on changes in physiologic condition or nutrient supply.[3] Nevertheless, current data are quite limited relative to information that has been accumulated on proteins' other qualities.[4]

Neutrophil cells, which are readily available, represent vital cell systems, the activity of which is highly affected by the whole organism's status.[5] Neutrophil cell mass is one of the major products of bone marrow, an organ which weighs approximately 2.6 kg and accounts for 4.5% of total body mass. Physiologic status highly affects leukocyte metabolism in vitro[6,7] and in vivo.[8] Neutrophils appear to be suitable for assessing protein turnover as affected by macronutrients and micronutrients. Nutrition, in turn, is an important factor for establishing physiologic

Address correspondence to Dr. Yosef Dror, Institute of Biochemistry, Food Sciences and Nutrition, Faculty of Agriculture and Environmental Quality Sciences, The Hebrew University of Jerusalem, Rehovot 76100, Israel.
This study was partially supported by grant no. 3825 of the State of Israel, Ministry of Health.
Received July 14, 1998; accepted April 26, 1999.

655 Avenue of the Americas, New York, NY 10010
0955-2863/99/$–see front matter
PII S0955-2863(99)00030-3

status, and each nutrient plays a distinct role in the metabolic pathways.

Vitamin B_6 is associated with a greater variety of reactions than most other vitamins. Its vitamer, pyridoxal 5′-phosphate (PLP), is a coenzyme of more than 150 enzymes that belong to at least five enzyme categories,[9] including most of the aminotransferases.[10] The vitamin B_6 needs of the elderly tend to be higher than those of young adults[11] and the former's vitamin B_6 status is very heterogeneous.[12] Therefore, a study of the effect of moderate vitamin B_6 supplementation in the elderly is of particular interest.[13] We studied the effect of vitamin B_6 supplementation on degradation rates of short-lived proteins in the neutrophils in a group of elderly subjects with a relative marginal deficiency. We assumed that vitamin B_6 supplementation would change the turnover rates of the neutrophil proteins. A biochemical model of the mechanism of cellular protein turnover as affected by nutritional intervention in human neutrophils is demonstrated.

Methods and materials

Subjects and scoring

After exclusion according to a set of categories defined in a medical chart, 50 apparently healthy and well-nourished institutionalized elderly subjects (18 males and 32 females) aged over 80 years were screened for vitamin B_6 status. The group of subjects, most of whom were living independently, were cooperative, were in good cognitive state, and included non-vitamin users and nonsmokers. Vitamin B_6 status was evaluated by a score consisting of (1) vitamin B_6 intake assessed by a dietary questionnaire and a customized food database[14,15]; (2) plasma pyridoxamine (PM) level; and (3) erythrocyte activation coefficient of aspartate aminotransferase (AC-AST). Each subject's vitamin status was ranked (the first two parameters were ranked from lower to higher values; the third was ranked from higher to lower) and then evaluated according to individual average rank of the three parameters. Subjects with the lowest scores ($n = 24$; 8 males and 16 females) were enrolled in the study. Blood (2 mL) was withdrawn from the brachial vein after an overnight fast and mixed with 40 USP heparin (Sigma Chemical Co., St. Louis, MO USA). For protein degradation measurements, blood was kept at room temperature; for AC-AST and plasma vitamers, it was kept on ice. The study was approved by the Kaplan Medical Center Committee on Human Experimentation. Subjects gave written consent after being fully informed of the nature of the study and procedures involved.

Activation coefficient of AST

AC-AST assay[16] was modified, and the hemolysis and preincubation conditions were changed.[17] For nonstimulated activity, heparinized blood was hemolyzed 2 to 4 hours after withdrawal in a solution (pH 7.5) containing Tris 40 mM, Na_2EDTA 2 mM, and digitonin (Merck, Darmstadt, Germany) 0.032 mM. For stimulated activity, blood was hemolyzed in the same solution, which also contained PLP 0.83 mM. Both hemolysates were kept at 4°C for 30 minutes, and then 10 μL of each was mixed with 0.65 mL of a solution (pH 8.0) containing Tris 32 mM, Na_2EDTA 1.6 mM, digitonin 0.026 mM, L-aspartic acid 200 mM, α-ketoglutarate 1.6 mM, NADH 0.2 mM, and malic dehydrogenase (Sigma) 0.24 IU/mL. Each mixture was kept at 30°C for 4 minutes; thereafter, the Δ340 nm was read. AC-AST was calculated as PLP-stimulated activity/nonstimulated activity (Δ340 nm).

B_6 vitamer determination

Plasma B_6 vitamers (pyridoxal, pyridoxine, pyridoxamine, pyridoxal phosphate, and pyridoxamine phosphate) and 4-pyridoxic acid were determined by high performance liquid chromatography[18] after plasma deproteinization by metaphosphoric acid 1.7% (w/v, final concentration).

Vitamin B_6 supplementation

Subjects were supplemented with vitamin B_6 (10 mg/d pyridoxine-HCl) for 28 days. Protein synthesis and degradation, as well as AC-AST value, were measured at baseline and following vitamin B_6 supplementation.

Neutrophil separation and incubation

Buffer The buffer used was Tris 50 mM and NaCl 154 mM, pH 7.4. Neutrophils were separated and incubated for degradation measurements essentially as described elsewhere.[19] Neutrophils were prepared from heparinized blood (140 USP units/mL) within 1 hour of withdrawal. No decrease in vital cell count was observed after the total 6-hour incubation.

Neutrophil separation A mix of 1 mL blood and 1 mL buffer was layered onto 1 mL histopaque 1083[20] (Sigma), which was itself layered on 1 mL histopaque 1119, and centrifuged at 30 × g (0.5 K rpm) for 10 minutes and at 500 × g (2 K rpm) for 5 minutes (swinging rotor, 140 mm at the bottom of the polystyrene conical test tube, 25–30°C). The leukocyte layer (above the erythrocytes) was sucked off and mixed with 2 mL of buffer; this was layered onto 1 mL of histopaque 1083, which was itself layered on 1 mL histopaque 1119, and centrifuged at 2,000 × g (4 K rpm) for 10 minutes; the neutrophil layer was sucked off and centrifuged at 2,000 × g for 10 minutes.

Incubation for synthesis and degradation measurements Synthesis was measured after 70 minutes (30 minutes of ^{35}S-Met incorporation, 10 minutes of centrifugation at room temperature, and a 30-minute washout). Degradation was measured after an additional 5-hour incubation period. Neutrophil incubation and washout were performed in a 1.5 mL Eppendorf test tube kept in a water bath at 37°C (the test tubes were inverted six times per minute). For the synthesis measurement, a mix of the neutrophil pellet, 25 μL of Dulbecco's Modified Eagle's medium (DMEM), and 20 μCi of L-^{35}S-Met (Amersham, Buckinghamshire, UK) was incubated for 30 minutes. The suspension was mixed with 0.5 mL of buffer and centrifuged at 2,000 × g for 10 minutes. The cell pellet was washed out for 30 minutes in 0.5 mL of DMEM containing methionine 0.15 mM and the suspension was divided into two equal volumes. The half for synthesis was mixed with 0.5 mL of cold buffer and centrifuged at approximately 8,000 × g for 5 minutes at 4°C; the cell pellet was mixed with 25 μL of buffer and kept at −20°C. The half for degradation was mixed with 0.66 mL of serum supplemented with amino acids,[19] and then was incubated for an additional 5 hours; it was mixed with 0.5 mL of cold buffer, centrifuged at approximately 8,000 × g for 5 minutes at 4°C, and the cell pellet was mixed again with cold buffer and centrifuged; finally, the cell pellet was mixed with 25 μL of cold buffer and kept at −20°C. Before protein separation, 25 μL treatment buffer [tris 50 mM, pH 6.8; sodium dodecyl sulphate (SDS) 4%; glycerol 20%; 2-mercaptoethanol 10%; and bromphenol blue 0.1 mg/mL] was added to the cell pellet, and the test tube was kept in boiling water for 90 seconds.

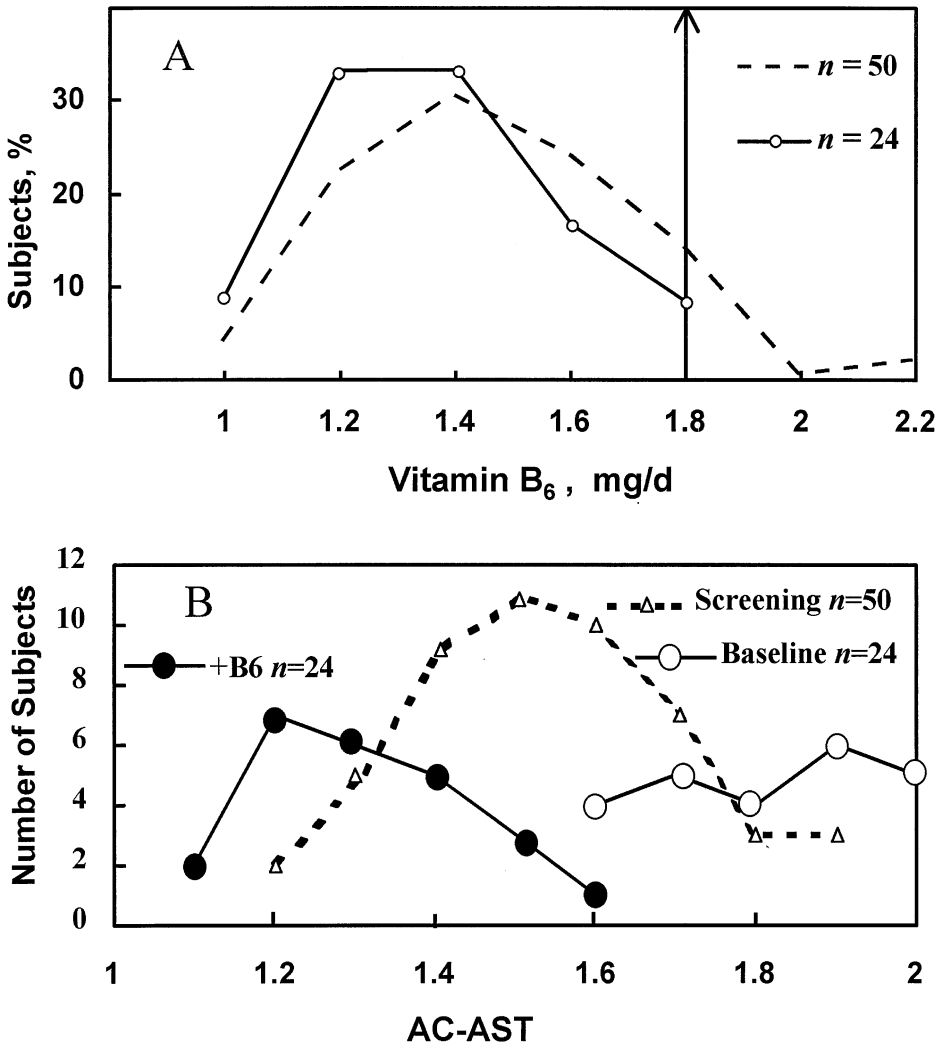

Figure 1 *(A)* Dietary vitamin B_6 intake of the screened ($n = 50$) and studied ($n = 24$) subjects. The arrow indicates the average recommended daily allowance (men and women). *(B)* AC-AST (activation coefficient of aspartate aminotransferase) values of the screened population ($n = 50$) and of the studied subjects ($n = 24$) as affected by vitamin B_6 supplementation ($+B_6$) for 28 days.

Protein separation and calculation of synthesis and degradation

After protein separation on polyacrylamide gel electrophoresis (PAGE; SDS 10%; 130 mm length, 1.5 mm thickness),[19] gels were placed between cellophane films and dried in an air gel-dryer for approximately 2 hours. The dried gel was exposed under an imaging plate for approximately 20 hours, and the imaging radioactivity was read by Fujix Bas1000 (Fuji Photo Film Co., Tokyo, Japan) and analyzed by Gel-Pro analyzer package software (Media Cybernetics, Silver Spring, MD USA). Each plate contained eight lanes, each of which was 14 mm (70 pixels) wide. When necessary, lanes were adjusted to be parallel by the software. A 35-by-600 pixel rectangle from the middle of a lane was electronically processed. We obtained 600 bands using this procedure. The relative synthesis rate of one band was calculated as the average value of 35 horizontal pixels. Each pixel (200-by-200 μm) may be characterized by 256 numerical values. Synthesis values were arbitrary, because the incorporation procedure lasted more than a couple of minutes (consequently, some degradation occurred). The lowest molecular weight of the separated proteins was approximately 22 kDa. Degradation was measured after an additional 5-hour incubation and the radioactive moiety was detected. The degradation rate of each of the 600 bands was calculated as follows: [(incorporated radioactivity) − (radioactivity after 5 hr)]/(incorporated radioactivity).When calculating $t_{1/2}$ (hours), we assumed a stable degradation rate during the 5-hour incubation. Therefore, $t_{1/2} = (5 \times \ln 2)/(\text{degradation rate during 5 hr})$.

The difference in degradation rates between baseline and vitamin B_6 supplementation was statistically analyzed using a two-tailed paired Student's *t*-test.

Results

Dietary vitamin B_6, AC-AST, and plasma B_6 vitamers

Vitamin B_6 intake of both the screened ($n = 50$) and studied ($n = 24$) populations displayed a normal distribution (*Figure 1A*). Most of the subjects were consuming less than the recommended daily allowance (RDA).[21] AC-AST values decreased significantly following vitamin B_6 supplementation (*Figure 1B*).

Plasma PLP of the unsupplemented ($n = 50$) subjects comprised approximately 50% of the total plasma vitamin B_6 (*Table 1*). The differences in plasma vitamers between females and males were nonsignificant. For the evaluation of vitamin status, coefficients of correlation were calculated

Table 1 Vitamin B_6 intake, erythrocyte AC-AST, plasma B_6 vitamers, and pyridoxic acid of unsupplemented subjects

	n	Vitamin B_6 intake, (mg/d)	AC-AST	Plasma concentration (nM)						
				PLP	PMP	PL	PN	PM	Total	4-PA
Males	18	1.4	1.44	48	5.9	23	8.1	16	101	4.6
Females	32	1.38	1.45	67	4.1	21	13	20	125	3.4
Av	50	1.39	1.44	60	4.8	21.9	10.9	18.7	116	3.8
STD		0.27	0.16	35	3.1	13	11	9.9	52	2
Min		1.02	1.16	12	0.3	6.4	0	0.1	24	17
Max		2.2	1.82	196	11	50	43	37	223	3
% of total B_6 vitamers				52	4.1	19	9.4	16	100	3.3

AC-AST–activation coefficient of aspartate animotransferase. PLP–pyridoxal phosphate. PMP–pyridoxamine phosphate. PL–pyridoxal. PN–pyridoxine. PM–pyridoxamine. 4-PA–4-pyridoxic acid.

between plasma vitamers and either vitamin B_6 intake or erythrocyte AC-AST. Coefficients between plasma PM and B_6 intake ($r = 0.33$) and between plasma PM and AC-AST ($r = -0.28$) were statistically significant ($P < 0.05$). Coefficients between either plasma PLP and vitamin B_6 intake ($r = -0.23$) or between plasma PLP and erythrocyte AC-AST ($r = -0.18$) were nonsignificant. Coefficients between the other plasma vitamers and the two parameters were nonsignificant as well. Therefore, plasma PM was used as one of the parameters for the evaluation of vitamin B_6 status.

Protein synthesis and degradation evaluations

A pair of typical lanes (after SDS-PAGE separation) of radioactive neutrophil proteins (from one neutrophil sample) is shown in *Figure 2* (synthesis, *Figure 2A*; degradation, *Figure 2B*). Whenever a band's value after degradation was higher than that of its synthesis counterpart, or whenever either of them was smaller than 0.01 (the arbitrary average synthesis value of one band was 0.3), degradation rate was not calculated. Thus, 15.9% of the total number of the bands were omitted. Because technical problems prevented the protein separation of five of the blood samples, only 19 paired neutrophil samples (baseline and after vitamin B_6 supplementation) were statistically analyzed. Merging the unpaired neutrophil samples into the statistical analysis resulted in a lower significance level of the $+B_6$/baseline ratios between degradation rates.

Protein synthesis

Synthesis rate was calculated by quantifying the radioactivity of each of the 600 protein bands. In almost all of the bands, incorporation of the radioactivity was significantly higher ($P < 0.01$) after vitamin B_6 supplementation (*Figure 3A*); only a few bands (15–29, 564–600) exhibited a nonsignificant increase. The ratios between synthesis rates ($+B_6$/baseline) were 3 to 6 for the first 13 bands and 1.6 to 2.8 for bands 14 through 414. From bands 415 to 600, there was a continuous fluctuating decrease down to 1.2.

Correlation between AC-AST and synthesis values

The average synthesis rates of the 600 bands from each neutrophil sample were plotted against AC-AST values measured in the same blood sample ($n = 38$). Higher AC-AST values correlated with lower average synthesis rates ($r = -0.70$, $P < 0.01$; *Figure 3B*). For the theoretical extreme value of AC-AST = 1 (highest vitamin B_6 status), the calculated average synthesis rate was 0.5, whereas for AC-AST = 2 (low vitamin B_6 status), it was 0.16 (as mentioned above, the arbitrary average synthesis value of one band was 0.3, a value close to the calculated average

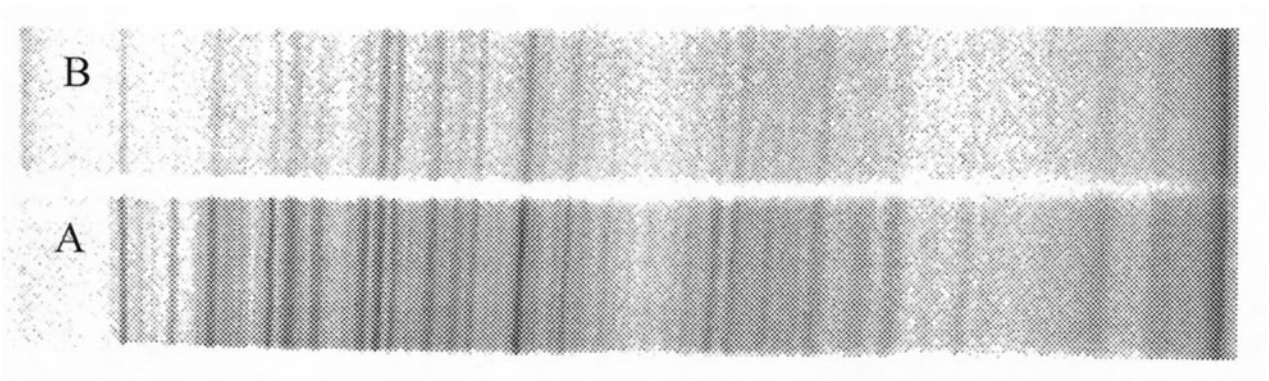

Figure 2 Radioactive image of two typical lanes after protein separation on polyacrylamide gel electrophoresis (PAGE) 10%. *(A)* After incubation for synthesis measurement. *(B)* After further incubation for degradation measurement.

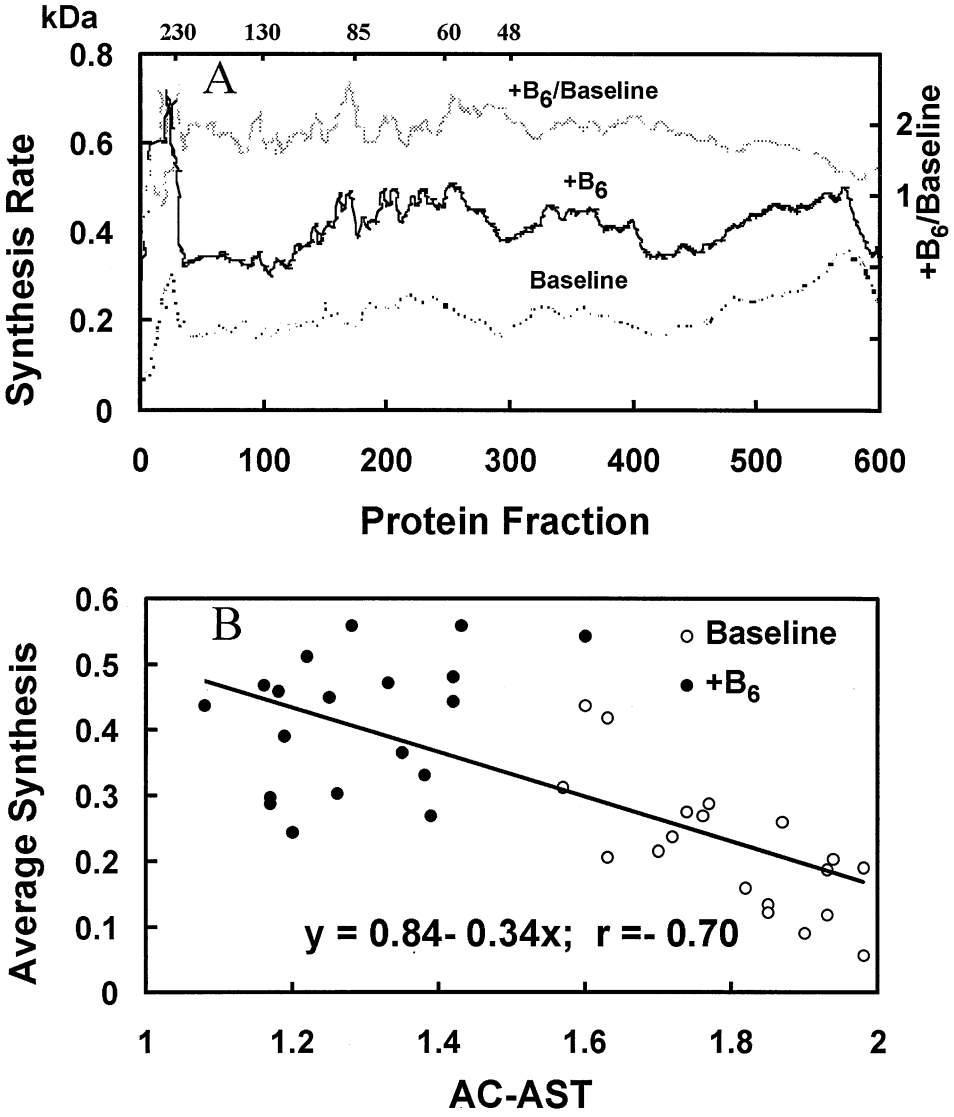

Figure 3 *(A)* Relative protein synthesis of the 600 protein bands (>250 kDa to <48 kDa) at baseline and after vitamin B_6 supplementation ($+B_6$), and the ratio $+B_6$/baseline ($n = 19$). *(B)* Correlation between AC-AST (activation coefficient of aspartate aminotransferase) values and the average synthesis rates in each neutrophil sample ($n = 38$).

values of 0.5 and 0.16). When plotted separately (baseline or after supplementation), although baseline neutrophil samples showed a similar correlation ($r = -0.75$, $P < 0.01$), samples obtained after vitamin B_6 supplementation displayed no correlation.

Vitamin B_6 supplementation and protein degradation

Most of the degradation rates decreased after vitamin B_6 supplementation (*Figure 4A*). The $t_{1/2}$ range (an average of 19 samples of each protein band) was 5 to 22 hours for baseline and 9 to 32 hours for vitamin B_6 supplementation (*Figure 4A*). Protein bands were classified according to the significance level of the effect of vitamin B_6 supplementation (*Figure 4B*).

Correlation between AC-AST values and average degradation rates

There was a positive correlation ($r = 0.51$, $P < 0.01$) between AC-AST values and the average degradation rates of the 600 bands of each neutrophil sample (*Figure 5*). When plotted separately, baseline neutrophil samples showed a similar correlation ($r = 0.40$, $P < 0.05$), whereas those after vitamin B_6 supplementation displayed no correlation.

Comprehensive evaluation of the effect of vitamin B_6 status (measured by AC-AST) on protein degradation

For this evaluation three vectors were calculated:

1. Ratios between degradation rates (average $+B_6$/average baseline) of each of the 600 protein bands (*Figure 4B*)
2. Coefficients of correlation between AC-AST values ($n = 38$) and degradation rates of each of the 600 bands
3. Same calculation as 2 above, except that random values ranging from 1 to 2 (as the range of AC-AST values) were assigned instead of the original AC-AST values

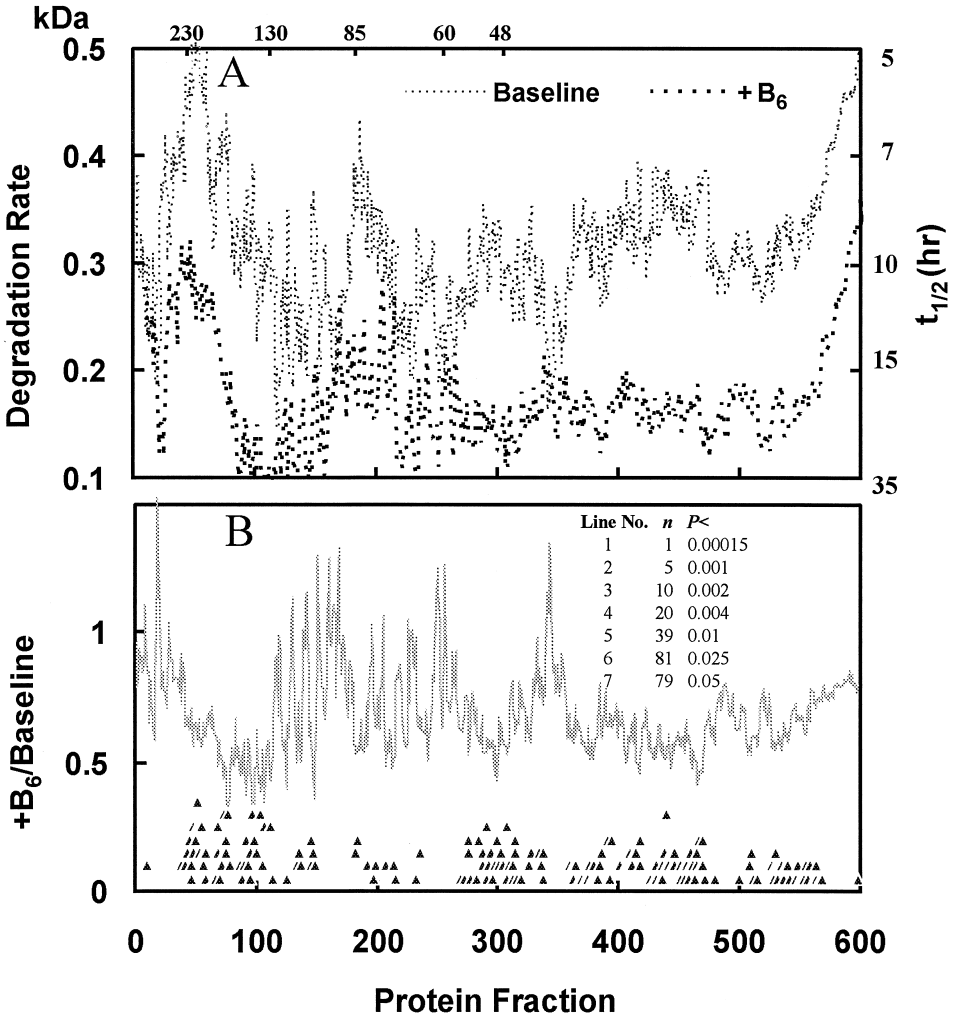

Figure 4 *(A)* Protein degradation rates of the 600 protein bands (>250 kDa to <48 kDa) at baseline and after vitamin B_6 supplementation ($+B_6$) (n = 19); the right axis indicates an estimation of $t_{1/2}$ (hours). *(B)* The ratios between degradation rates ($+B_6$/baseline). The seven lines of triangles at the bottom designate the significance level of the change in degradation rates after vitamin B_6 supplementation (the upper line indicates the highest significance level). The table inside the frame indicates the line number of the triangles (Line No.), the number of protein bands in each triangle line (n), and the significance level ($P<$).

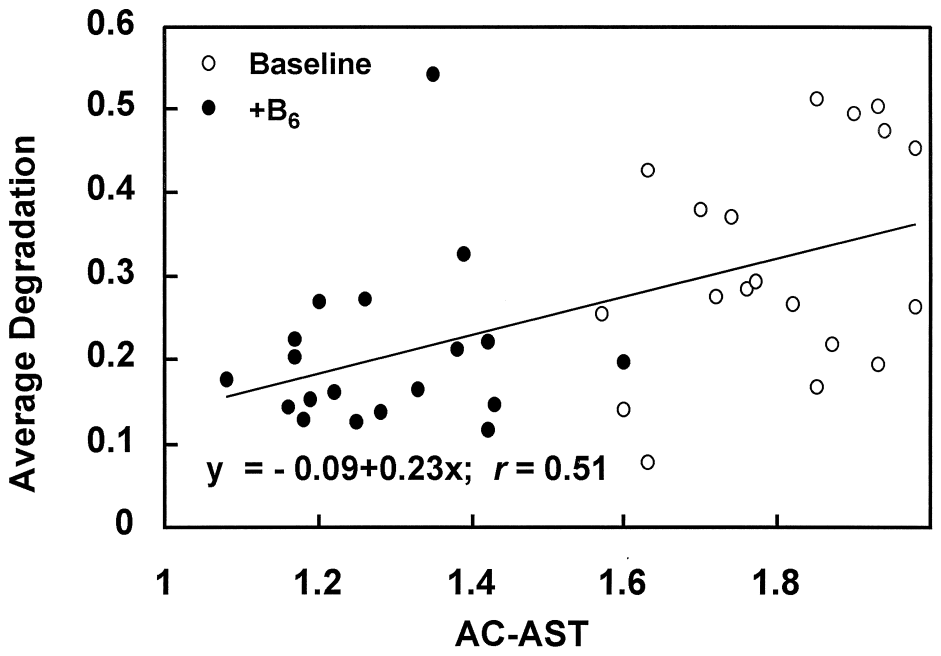

Figure 5 Correlation between AC-AST (activation coefficient of aspartate aminotransferase) values and the average degradation rates in each neutrophil sample (n = 38).

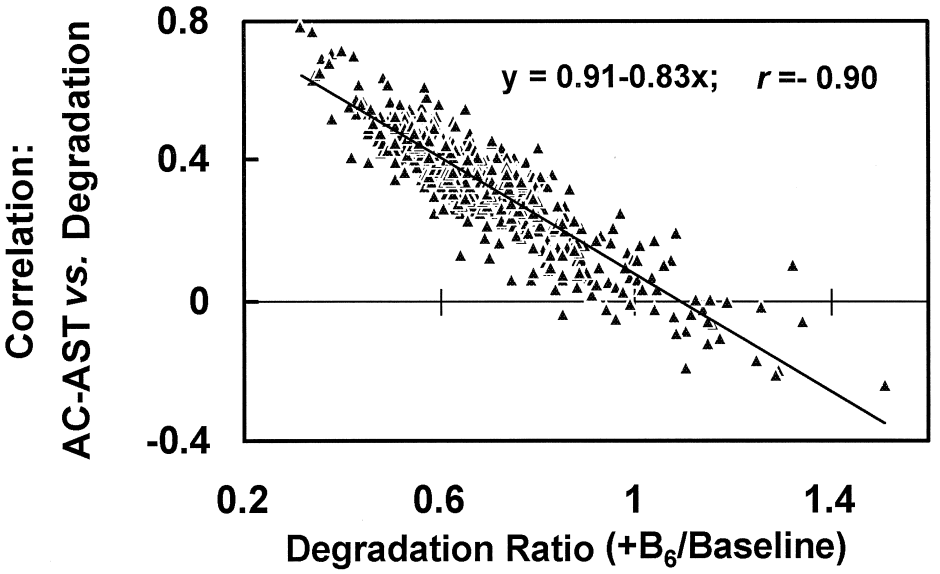

Figure 6 A plot between two vectors: (1) ratios between degradation rates ($+B_6$/baseline) and (2) coefficients of correlation between AC-AST (activation coefficient of asparatate aminotransferase) values and the degradation rates of the 600 protein bands of each neutrophil sample. There is a low probability ($P < 0.001$) for getting a high coefficient of correlation ($r > | 0.65 |$) when random numbers instead of AC-AST values (12,500 calculations) are assigned.

(however, any range of random numbers show the same results).

A high negative correlation was found between the first and second vector values (*Figure 6*). To rule out the possibility of internal correlates (because vector 2 was produced by calculation of coefficients of correlation, and because some protein bands' data are missing), random numbers[22] (vector 3) rather than AC-AST values were assigned. There is a low probability ($P < 0.001$) for getting a high (negative) coefficient of correlation when random numbers are assigned. Fractions 110 to 159 were most affected by vitamin B_6 status (as evaluated by AC-AST).

Discussion

The effect of vitamin B_6 supplementation on AC-AST (one of the biomarkers of vitamin B_6 status) indicated that the vitamin B_6 status of the studied population was relatively low. After vitamin B_6 supplementation for 4 weeks at approximately five times the RDA, the AC-AST values decreased significantly. Some subjects had higher AC-AST values at baseline than during the screening period. The discrepancy may be explained by seasonal effect. To obtain a substantial vitamin effect on the biochemical parameters, we selected subjects with the lowest vitamin B_6 status. Because vitamin status is more accurately defined using several measures, scores of three parameters were calculated (intake, AC-AST, and a vitamer concentration). Plasma PLP or total vitamer concentrations are usually considered indices for vitamin B_6 status.[23] However, we chose plasma pyridoxamine level as one of the score's components (to evaluate vitamin B_6 status), because the highest coefficient of correlation was found between AC-AST and pyridoxamine values. As stated in a recent review,[24] there is little information, about the relative rates of pyridoxine, pyridoxamine, and pyridoxal metabolisms. The higher response of mouse tissue pyridoxamine phosphate versus pyridoxal to dietary pyridoxine[25] may support our approach.

We assume the impact of vitamin B_6 on protein metabolism to have been demonstrated by (1) a significant effect of AC-AST on average synthesis (*Figure 3B*) and degradation (*Figure 5*) values and (2) a clear negative correlation between the two vectors in *Figure 6*.

Validation of correlation by replacing AC-AST values with random numbers may enable us to evaluate the relationship between vitamin status and specific protein fractions. Following vitamin B_6 supplementation, the degradation rate of a specific protein band decreases concomitantly with an increase in the coefficient of correlation between AC-AST (higher value means lower vitamin status) and the degradation rate of a specific protein band. The degradation rates of proteins of the highest as well as the lowest molecular weights were similarly affected by vitamin status.

A change in the degradation rates of enzymes and other cellular proteins provides a key to understanding the mechanism of nutritional and physiologic effects on cellular metabolism, as well as on the whole organism. This change is probably the main determinant controlling the increase or decrease in cellular enzyme (and protein) concentration.[1] A specific effect of vitamin B_6 deficiency on the increase in the degradation rate of cystathionase, a pyridoxal phosphate-activated enzyme, has been already shown in rats.[26,27] A similar effect of a niacin-deficient diet on increasing in the degradation rates of pectoral muscle proteins has been shown in quails.[28] As opposed to the induced vitamin B_6 deficiency in those animals, in our study the effect of vitamin B_6 supplementation was shown in marginally deficient elderly subjects.

Vitamin B_6 supplementation significantly increased the synthesis rates of the short-lived neutrophil proteins in almost all of the bands and significantly decreased degradation rates in many of them. The degradation rates of 235 protein bands decreased significantly ($P < 0.05$). Degradation rates of 75 protein bands (which decreased at a significance level of $P < 0.01$) were classified according to percentage decrease (*Table 2*). In some bands, $t_{1/2}$ increased

Table 2 Decrease in degradation rates (% of baseline) following vitamin B_6 supplementation ($+B_6$), $P < 0.01$

Decrease in degradation rate (%)	Range of $t_{1/2\ (hr)}$		Band number
	Baseline	$+B_6$	
60–70	20–32	6–10	77, 78, 93, 98, 99, 105, 108
50–60	15–28	6–13	70, 74, 75, 76, 100, 101, 113, 148, 300, 417, 418, 444, 466, 467, 468, 470
40–50	10–26	5–13	49, 52, 53, 55, 56, 59, 69, 96, 145, 184, 185, 277, 285, 286, 289, 290, 291, 295, 303, 309, 328, 387, 410, 413, 416, 439, 440, 445, 447, 460, 510, 530
25–40	9–24	5–16	44, 45, 46, 50, 51, 54, 57, 137, 146, 235, 236, 276, 302, 315, 316, 329, 336, 338, 392, 396

approximately threefold, whereas in most of them the increase was approximately twofold. Degradation rates of some bands (11, 313, 335, and 337) decreased significantly ($P < 0.05$) by 14 to 19% (not shown). Our procedure enables us to detect, in humans, decreases of less than 20% in the degradation rates of distinct protein bands.

Proteins are heterogeneous in their life spans: some have a $t_{1/2}$ of 5 minutes, whereas others have a $t_{1/2}$ of 120 days or more. The mean $t_{1/2}$ of neutrophil proteins was 11 hours (range, 5–32 hours). A half-life of 5 hours is probably the lowest $t_{1/2}$ that can be evaluated by the incubation procedure employed in our study. A shorter $t_{1/2}$ might be determined using a higher concentration of ^{35}S-Met, a shorter incubation period (incorporation and washout), and better protein separation techniques. In some human tissues, the calculated $t_{1/2}$ values of proteins were as follows: whole body, 29 days[29,30]; liver, 4 days; stomach, 3 days; muscle, 30 to 69 days[31,32]; serum albumin, 10 days[33]; and lymphocytes, 6 to 11.5 days.[33–35] Degradation rates of only some of the neutrophil proteins were measured by our procedure. On the one hand, the radioactivity of the very short-lived proteins was washed out during the 30-minute washout incubation. On the other hand, the radioactivity of the long-lived proteins was obscured by that of short-lived proteins[36] (because most of the short-lived proteins are synthesized at a higher rate). Therefore, the latter, which probably comprise most of the regulatory proteins,[37] are predominantly determined.

When high molecular weight proteins are cleaved, their fragments may appear as low molecular weight proteins, which could obscure the unfragmented low molecular weight proteins. Therefore, the calculation of the degradation rates of some low molecular weight protein bands might be biased, but we assume that this effect is comparatively small.

Neutrophils are probably the most convenient cells for measuring nutritional effects on protein metabolism in humans because of their availability and life span. We defined our study as protein degradation in neutrophils, although the neutrophil fraction contained a negligible mass of lymphocytes. During incubation, all of the neutrophil samples (before and after supplementation) were maintained in DMEM containing the same pyridoxal concentration (10 μM). Thus, PLP probably affected the rates of protein synthesis and degradation during the neutrophil life cycle of approximately 14 days in bone marrow and approximately 6 hours in peripheral blood[38] (before the incubation procedure).

PLP protects in vitro tyrosine aminotransferase from tyrosine cleavage by conformational alteration[39] and protects other PLP-dependent apoenzymes in vivo from cellular degradation.[27,40] PLP binds to the active as well as other sites by ligation to lysyl[41] or to other moieties.[42–45] Therefore, PLP could affect the degradation of proteins that are not necessarily activated by PLP via Schiff base formation and ligation to specific regions of a primary sequence or to a specific motif of proteins.[46–48] Higher concentrations of PLP probably decrease the ubiquitination by producing Schiff bases with lysyl residues. Moreover, at least traces of covalently bound, labeled PLP have been detected in most of the protein fractions of skeletal muscles.[49] However, small changes in total PLP concentration could cause substantial changes in the amount of free PLP available for nonspecific binding. The effect of PLP on long-lived proteins is not necessarily the same as that on short-lived ones. In severe vitamin B_6 depletion, the activities of PLP-bound enzymes were remarkably affected. Whereas ornithine decarboxylase activity increased more than threefold,[50] probably due to a decrease in its degradation rate, serine transhydroxymethylase activity decreased over fourfold.[51]

PLP is a coenzyme of only a small part of all cellular proteins. One may wonder how vitamin B_6 affects degradation rates of so many protein fractions (bands), because the library of vitamin B_6-dependent enzymes is limited to only a few hundred.[44] One probable answer is the adherence to the N-end rule of protein degradation. Lysyl residues serve as N-degrons (a degradation signal) for ubiquitination of short-lived proteins prior to protein degradation in the proteasome.[52,53]

Here we suggest a tool for simultaneously examining changes in the degradation rates of many neutrophil protein fractions (bands), after in vivo supplementation of a specific nutrient. A similar approach of simultaneous degradation rate measurements of some protein fractions by separation on a PAGE has been shown in dog cardiac myocytes[54] and monkey kidney cells.[55] However, our procedure, which was performed in vitro, mirrors in vivo protein activity following nutritional intervention. Despite the comparatively high variation of degradation rates between subjects and samples, we determined (via statistical analysis) synthesis and degradation rates of many specific protein bands.

We show the feasibility of classifying protein fractions according to the extent of change (percent decrease) in degradation rates (*Table 2*) and according to the significance level of this change (*Figure 4B*). Such protein classifications might provide a tool for comparing the effects of various nutrients on protein degradation.

The procedure described in our study has some advantages. First, it measures degradation rates of many protein bands simultaneously and could potentially be adapted to detect degradation rates of single proteins. Second, the radioactive marker is used in vitro. Third, it could poten-

tially be used in human as well as animal experimentation, because for each assay only 1 mL of blood is needed. Finally, this methodology could potentially be used for measuring kinetic changes in degradation rates of protein bands as affected by nutritional or pharmacologic treatment, or any change in physiologic status.

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