Vitamin B₆ Metabolism in Chronic Alcohol Abuse

PYRIDOXAL PHOSPHATE LEVELS IN PLASMA AND THE EFFECTS OF ACETALDEHYDE ON PYRIDOXAL PHOSPHATE SYNTHESIS AND DEGRADATION IN HUMAN ERYTHROCYTES

LAWRENCE LUMENG and TING-KAI LI

From the Departments of Medicine and Biochemistry, Indiana University School of Medicine and Veterans Administration Hospital, Indianapolis, Indiana 46202

ABSTRACT The plasma pyridoxal-5′-phosphate (PLP) level of alcoholic subjects has been compared with that of non-alcoholic individuals in order to ascertain the incidence of abnormal vitamin B₆ metabolism in chronic alcohol abuse. 66 alcoholic subjects were selected on the basis that they did not exhibit abnormal liver function tests and hematologic findings. 35 of them had plasma PLP concentrations less than 5 ng/ml, the lowest value encountered in 94 control subjects, indicating a high incidence of deranged PLP metabolism in alcoholic patients even when hepatic and hematologic abnormalities are absent. The biochemical basis for the altered PLP metabolism in chronic alcohol abuse was examined. Low plasma PLP levels in alcoholics were not accompanied by decreased pyridoxal kinase and pyridoxine phosphate oxidase activities in erythrocytes. Further studies with erythrocytes demonstrated that the cellular content of PLP is determined not only by the activities of these PLP-synthesizing enzymes but also by the activity of a phosphate-sensitive, membrane-associated, neutral phosphatase, which hydrolyzes phosphorylated B₆ compounds.

Acetaldehyde, but not ethanol, impaired the net formation of PLP from pyridoxal, pyridoxine, and pyridoxine phosphate by erythrocytes. However, when the B₆-phosphate phosphatase activity was inhibited by 80 mM phosphate, this effect of acetaldehyde was abolished. By the use of broken cell preparations, it was possible to demonstrate directly that the action of acetaldehyde is mediated by the phosphatase, resulting in an acceleration of the degradation of the phosphorylated B₆ compounds in erythrocytes.

INTRODUCTION

Pyridoxal-5′-phosphate (PLP)[1] is the biologically active, coenzymic form of the vitamin B₆ compounds. Although the importance of this cofactor in metabolism is well established (1), relatively little is known about its involvement in disease processes. Deficiency of PLP has been causally implicated in several clinical disorders in the alcoholic patient, including peripheral neuropathy (2), convulsions (3), sideroblastic anemia (4), and liver disease (5). Of particular interest is that deprivation of vitamin B₆ produces fatty liver in experimental animals and deficiency of this vitamin in the monkey for prolonged periods results in florid cirrhosis (6, 7). In the rat, chronic alcohol feeding has been shown to act synergistically with vitamin B₆ deprivation to produce marked fatty infiltration of the liver (5). No human counterparts of these experimental lesions have been reported, but it is known that patients with severe alcoholic fatty liver and cirrhosis have decreased plasma PLP and hepatic vitamin B₆ content (8, 9).

Hines and Cowan recently studied three alcoholic patients, during and as long as 3 wk after the chronic administration of large amounts of alcohol and found that alcohol consumption caused a depression of the plasma concentration of PLP and impaired the conversion of pyridoxine to PLP (4). Hines has further

[1] *Abbreviations used in this paper:* Pi, inorganic phosphate; PLP, pyridoxal-5′-phosphate; TEA, triethanolamine hydrochloride.

reported in a preliminary communication that the pyridoxal kinase (ATP: pyridoxal 5'-phosphotransferase, EC 2.7.2.35) activities of red cells and hepatic tissue are reduced in some subjects during alcohol administration, and that kinase activity returns to normal after the cessation of alcohol intake (10). If these observations, in fact, represent a direct effect of ethanol on PLP synthesis, the incidence of deranged vitamin B_6 metabolism in alcoholic patients should be high, although this has not been specifically determined. These findings would further suggest that pyridoxal kinase is the rate-limiting enzyme in the synthesis of PLP in man and a major determinant of the concentration of PLP in plasma and tissues.

In this communication, we report the prevalence of abnormally low plasma PLP concentrations in patients with chronic alcohol abuse but without clinically detectable hepatic and hematologic disease. The metabolism of vitamin B_6 in normal human erythrocytes has been studied in order to define the optimal conditions for assay of the PLP synthesizing and degradative enzymes and to delineate their relative importance in the regulation of the intracellular content of PLP. The erythrocytic pyridoxal kinase and pyridoxine phosphate oxidase (pyridoxine phosphate: oxygen oxidoreductase, EC 1.4.3.5) activities in patients with chronic alcoholism who exhibit decreased plasma PLP levels have been measured. Lastly, the effects, in vitro, of ethanol and its oxidative product, acetaldehyde, on the net synthesis of PLP in human erythrocytes have been examined.

METHODS

Subjects. 66 male alcoholic patients, 18–68 years of age, comprised the experimental group. All were in-patients of the Alcoholism Treatment Unit of the Indianapolis V. A. Hospital, chosen because they had normal physical examinations and laboratory tests (including complete blood count, bromsulfalein excretion, serum albumin, bilirubin, alkaline phosphatase, glutamic-oxaloacetic transaminase, and prothrombin time). 18 had transiently elevated serum glutamic-oxaloacetic transaminase activities and 9, transiently increased serum alkaline phosphatase activities, both of which had returned to normal by the time of the study. Liver biopsies were not performed, since in no instance were they clinically indicated. All of the subjects had long-standing histories of chronic alcohol abuse (11), but had abstained from alcohol 1–5 days at the time of the study. 94 healthy male volunteers from the medical center personnel pool and individuals free of chronic and acute illnesses undergoing routine annual physical examinations constituted the control group. The dietary intakes of the control subjects were considered to be adequate by history, but those of the alcoholic patients could not be reliably assessed. Individuals taking vitamin supplements were excluded.

Preparation of plasma samples and erythrocytes. Venous blood was collected with 2 mM EDTA as anticoagulant. All specimens were protected from exposure to light in order to avoid photolysis of PLP. Plasma was separated

within 30 min of collection and stored at 4°C if assayed immediately or at −10°C if assayed within 2 days of collection. Stored in this manner, no significant loss of PLP from the plasma was observed for as long as 4 days. After separation from plasma and buffy coat, the erythrocytes were washed three times with 0.15 M NaCl at 4°C by centrifugation and resuspension. The cells were finally suspended in appropriate incubation media to packed cell volumes of 15%. Aliquots of the suspended cells were then added to the final incubation mixtures.

Preparation of supernatant and ghost fractions from washed erythrocytes. Previously washed and packed erythrocytes were hemolyzed in 10 vol of a 12 mM triethanolamine hydrochloride (TEA)-NaOH buffer (pH 7.4) at 4°C and allowed to stand for 10 min with intermittent mixing. The hemolysate was clarified by centrifugation at 30,000 g for 30 min and the supernatant fraction separated from the sedimented erythrocyte ghosts. For studies of the intracellular distribution of enzymes, the ghost fraction was washed six or more times with TEA buffer until the supernate became colorless. For other studies the red cell membrane fraction was washed twice. Where indicated, the erythrocyte ghosts were sonicated, employing a model 1000 Insonator (Savant Instruments, Inc., Hicksville, N. Y.) (20 kHz) supplied with a half-inch sonohorn. The reference meter setting was 75 and sonication was applied twice in 10-s pulses at 4°C.

Analyses. PLP in plasma samples and erythrocytic suspension were assayed enzymatically with tyrosine decarboxylase apoenzyme. The method described by Chabner and Livingston (12) was modified as follows: (*a*) Commercially available apoenzyme isolated from *Streptococcus fecalis* was further purified by $(NH_4)_2SO_4$ fractionation. Contaminating pyridoxal kinase activity was completely removed by precipitation in a 60% saturated $(NH_4)_2SO_4$ solution. The apoenzyme was then concentrated by precipitation with $(NH_4)_2SO_4$ at 85% saturation, and resolubilized by dialysis against 0.3 M sodium citrate buffer (pH 6.0) containing 24% (vol/vol) glycerol and 2 mM mercaptoethanol. This partially purified preparation was stable for as long as 6 wk at −10°C without significant loss of PLP-reconstitutable enzyme activity. (*b*) Deproteinized plasma samples were prepared by adding 1.0-ml aliquots to 0.10 ml of 75% (wt/vol) trichloroacetic acid. After centrifugation, the trichloracetic acid was extracted by water-saturated and peroxide-free ether, thereby obviating the problems of sample neutralization. (*c*) The assay mixture contained 0.15 M sodium citrate buffer (pH 6.0), 5 mM L-[1-^{14}C]tyrosine (sp act 20 μCi/mmol), apoenzyme, and 0–4 ng of PLP standard or unknown. Citrate provided a greater buffering capacity than did acetate at pH 6.0. A higher concentration of tyrosine gave higher and more reproducible reconstituted enzyme reaction rates. With these modifications, 90±8.5% of the PLP could be recovered and reproducibility of the method was ±4.7% (1 SD). All assays were performed in duplicate. In the experiments in which ethanol or acetaldehyde was employed, these compounds in the concentrations used did not interfere with this assay method for PLP.

Pyridoxal was assayed fluorometrically, after separation by ion-exchange chromatography and oxidation in the presence of cyanide (13). Hemoglobin was determined as cyanmethemoglobin (14).

Incubation conditions. All experiments were performed in either 10-ml or 25-ml Erlenmeyer flasks incubated in a shaking waterbath at 32°C. The reaction flasks and samples were protected from ordinary room light by the

use of darkened rooms and yellow fluorescent lights in order to avoid photolysis of PLP. The incubation flasks were sealed by rubber caps when acetaldehyde or ethanol was employed. Reactions were initiated by adding the appropriate substrates and terminated at different time intervals by transferring 1.0-ml aliquots into tubes which contained 0.10 ml of 75% trichloracetic acid. Pyridoxal kinase activity was determined by measuring the formation of PLP from pyridoxal and ATP. Pyridoxine phosphate oxidase activity was measured by following the formation of PLP with pyridoxine phosphate as substrate. The activity from the coupled kinase and oxidase reactions was determined by measuring the formation of PLP with pyridoxine and ATP as substrates. The B_6-phosphate phosphatase activity was determined by the formation of pyridoxal from PLP. The optimal conditions for the assay of these enzymes in human erythrocytes have not been reported previously and hence have been specifically determined for both the intact cells and hemolysate supernatant fractions as part of this study.

Reagents. Pyridoxal, pyridoxine, PLP, and tyrosine decarboxylase apoenzyme were purchased from Sigma Chemical Co., St. Louis, Mo. Pyridoxine phosphate was obtained from Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y. The concentrations of the vitamin B_6 compounds were determined by their molar absorptivity in 0.1 N NaOH (15). Acetaldehyde was redistilled immediately before use.

RESULTS

Plasma PLP concentrations of control subjects and alcoholic patients. The plasma PLP levels of control subjects were found to be remarkably constant as a function of time. Repeated measurements in two subjects over periods of as long as 6 mo did not show coefficients of variation beyond 10% (Fig. 1). A similar finding was obtained in four other subjects measured at weekly intervals for 2 mo. Meals did not alter these values significantly. However, person-to-person variation of the concentration of plasma PLP was considerable, and there was an age-related decline of the mean plasma
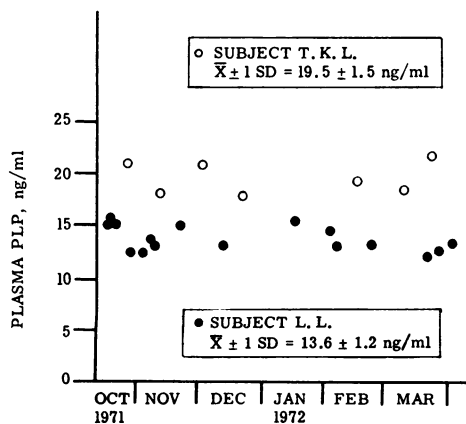

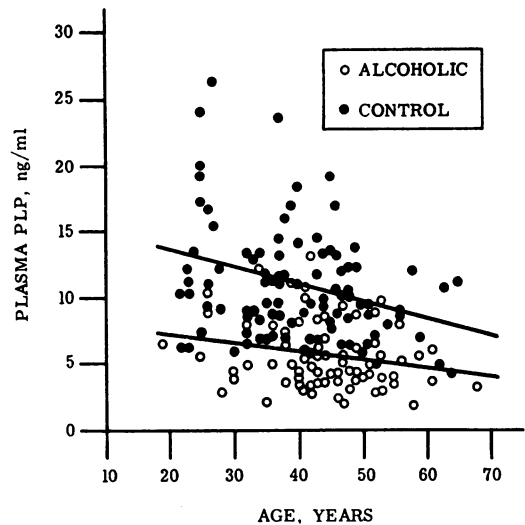FIGURE 1 Serial measurements of the plasma PLP concentration of two control subjects.

FIGURE 2 Plasma PLP concentration of alcoholic and nonalcoholic males, plotted as a function of age. The upper line is the regression line of the control group and the lower, of the alcoholic group.

PLP concentration (Fig. 2). In the 94 male control subjects, plasma PLP concentrations ranged from 5 to 26.3 ng/ml. This range of values for a normal population is in agreement with that reported previously (12, 16). In contrast, the plasma PLP concentrations of 66 alcoholic male individuals free of hematologic and liver disorders ranged from 1.9 to 13.1 ng/ml. 35 of the alcoholic subjects exhibited values below 5 ng/ml. The slopes of the regression lines for the control and alcohol groups were not significantly different, indicating that the age-related decline of mean plasma PLP was comparable in the two groups. However, the age-adjusted mean plasma PLP concentration of the alcoholic group was 4.7 ± 0.6 ng/ml below that of the control group, a highly significant difference ($P < 0.001$). These data clearly establish that alcoholic subjects, even when free of hematologic or hepatic disease, very commonly have lowered levels of plasma PLP.

Studies on the metabolism of PLP in normal human erythrocytes. Pyridoxal kinase, pyridoxine phosphate oxidase, and B_6-phosphate phosphatase activities have been described in human erythrocytes (17, 18). The first two enzymes catalyze the synthesis of PLP whereas the third enzyme catalyzes the hydrolysis of phosphorylated vitamin B_6 compounds. When intact erythrocytes were incubated in media containing either 1.5 or 80 mM inorganic phosphate (Pi), they converted pyridoxal, pyridoxine, and pyridoxine phosphate to PLP. However, in the presence of 80 mM Pi, the rates of net synthesis of PLP from all three substrates were considerably higher and more constant than those in the presence of 1.5 mM Pi (Fig. 3). These findings are in
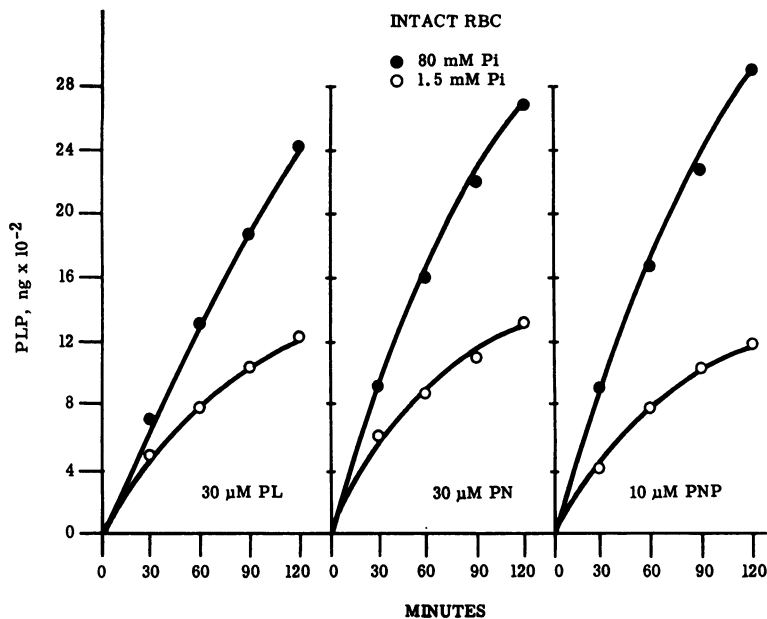
FIGURE 3 Effect of 1.5 and 80 mM Pi on the conversion of vitamin B_6 compounds to PLP in intact erythrocytes. The low Pi medium contained 50 mM TEA (pH 7.4), 1.5 mM sodium phosphate (pH 7.4), 10 mM KCl, 102 mM NaCl, and 5 mM glucose. The high Pi medium contained 50 mM TEA (pH 7.4), 80 mM sodium phosphate (pH 7.4), 10 mM KCl, 7 mM NaCl, and 5 mM glucose. Washed erythrocytes (RBC) equivalent to 215 mg hemoglobin were used. Reactions were started by adding the B_6 substrates, 30 μM pyridoxal (PL), 30 μM pyridoxine (PN) and 10 μM pyridoxine phosphate (PNP). Each point represents the mean of duplicate determinations.

agreement with those reported by Anderson, Fulford-Jones, Child, Beard, and Bateman (17).

To discern the intracellular location of pyridoxal kinase, pyridoxine phosphate oxidase, and B_6-phosphate phosphatase, erythrocytes were hypotonically lysed and the ghosts separated by centrifugation. Whereas the kinase and oxidase activities were located exclusively in the supernatant fraction of the hemolysate, B_6-phosphate

TABLE I

Distribution of Pyridoxal Kinase, Pyridoxine Phosphate Oxidase, and B_6 Phosphate Phosphatase Activities in Human Erythrocytes

Preparation	Kinase	Oxidase	Phosphatase
	$\mu g\ PLP/h/g\ Hb$	$\mu g\ PLP/h/g\ Hb$	$\mu g\ PL/h/g\ Hb$
Intact erythrocytes	10.5 ± 0.5	6.5 ± 0.2	—
Hemolysate supernate	8.6 ± 0.3	4.2 ± 0.2	0
Sonicated ghosts	0	0	4.4 ± 1.0

The kinase and oxidase activities in washed erythrocytes were assayed in the 80 mM Pi medium as described for Fig. 3. The reaction mixtures for assaying the kinase and oxidase activities in hemolysate supernate and sonicated ghosts contained 10 mM TEA, 1 mM $MgCl_2$, 80 mM potassium phosphate, and hemolysate supernate or sonicated ghosts derived from 1 ml of packed cells in final volumes of 10 ml. For the kinase reaction, 2.5 mM ATP and 30 μM pyridoxal were added as substrate and pH was 8.0. For the oxidase reaction, 10 μM pyridoxine phosphate was substrate and pH was 7.4. The phosphatase activities were assayed in a reaction mixture which contained: 10 ml of 20 mM TEA buffer (pH 7.1); 2 ml of 10 mM $MgCl_2$; 1.0 μg/0.5 ml of PLP; 3.2 ml of 6 M KCl; 1.0 ml of sonicated ghosts prepared from 0.25 ml of packed erythrocytes; and 3.3 ml of water. 1 ml of packed erythrocytes contained 280 mg hemoglobin (Hb). Values are means$\pm$SE of four experiments.
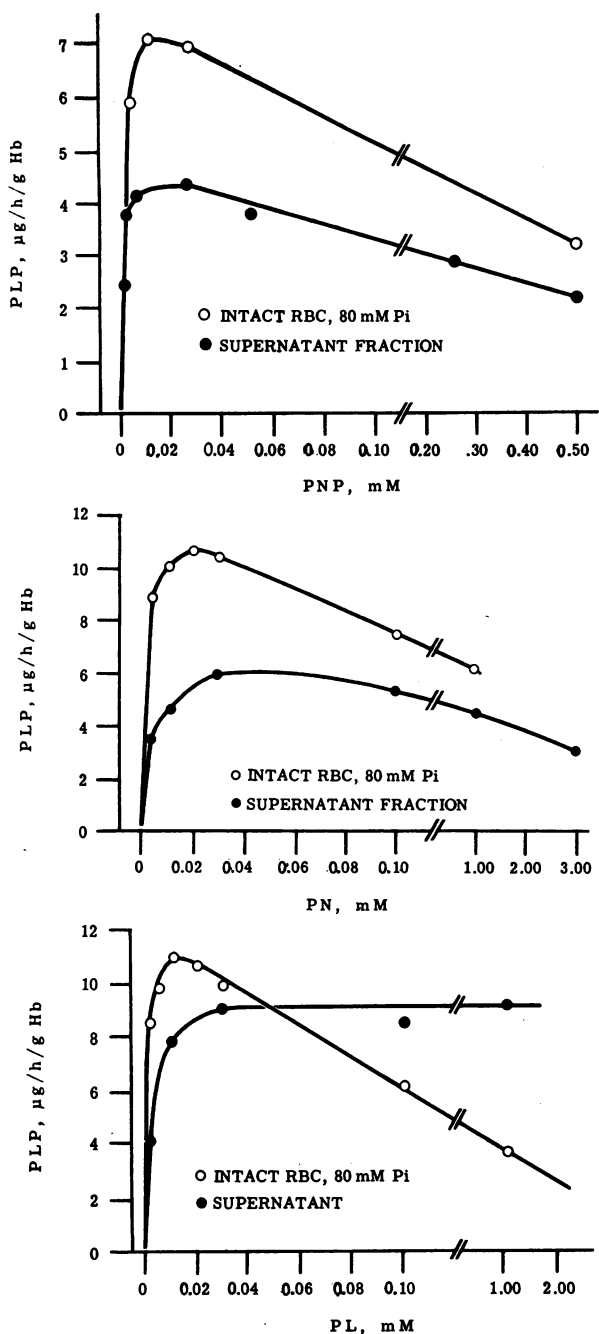
FIGURE 4 Effect of substrate concentration on the rate of conversion of pyridoxal (PL), pyridoxine (PN) and pyridoxine phosphate (PNP) to PLP. The 80 mM Pi medium as described in Fig. 3 was used for intact cells. The incubation medium for studying the phosphorylation of pyridoxal in hemolysate supernate contained 10 mM TEA (pH 8.0), 80 mM potassium phosphate (pH 8.0), 1 mM MgCl₂, 2.4 mM ATP and hemolysate supernate to a final hemoglobin concentration of 1.30 g/100 ml. The medium for studying the conversion of pyridoxine phosphate to PLP was as described above except that ATP was omitted and the final hemoglobin concentration was 1.67

phosphatase activity was found to be associated principally with the erythrocyte membrane (Table I). Although erythrocyte membranes are known to contain an alkaline phosphatase of broad substrate specificity, the pH optimum for the hydrolysis of PLP was found to be 7.1, suggesting that the reaction is catalyzed by a separate enzyme. This activity was increased by sonication and inhibited by high concentrations of Pi, e.g., 80 mM Pi. In contrast, Pi did not influence the activities of pyridoxal kinase and pyridoxine phosphate oxidase in the hemolysate supernate. The higher rates of the net PLP synthesis observed in intact erythrocytes when exposed to a high concentration of Pi can thus be explained by the inhibitory action of Pi upon the neutral B₆-phosphate phosphatase activity of the erythrocyte membranes.

Studies on the optimal conditions for the assay of pyridoxal kinase and pyridoxine phosphate oxidase in intact human erythrocytes. Since 80 mM Pi was effective in inhibiting B₆-phosphate phosphatase, the activities of pyridoxal kinase, pyridoxine phosphate oxidase, and their coupled reactions could be assayed in intact red cells by incubation in the presence of 80 mM Pi. It was found that all reactions in intact red cells were subject to substrate inhibition (Fig. 4). The optimal substrate concentrations for pyridoxal, pyridoxine phosphate, and pyridoxine were 10–30 μM.

Studies on the optimal assay conditions and other enzymatic properties of the pyridoxal kinase and pyridoxine phosphate oxidase in hemolysate supernatant fractions. In assays of the membrane-free, supernatant fractions of hemolysates, patterns of substrate inhibition similar to those for the intact cells were found for pyridoxine and pyridoxine phosphate, but substrate inhibition was not observed with pyridoxal as the substrate (Fig. 4). Several other enzymatic properties of pyridoxal kinase and pyridoxine phosphate oxidase studied are of interest, particularly in regard to the assay of their activities in hemolysate supernates. Zn²⁺ could replace Mg²⁺ as the divalent cation in the kinase reaction. Mn²⁺ and Co²⁺ were substantially less effective that Zn²⁺ or Mg²⁺, whereas Ni²⁺ and Fe²⁺ were inactive. When added to the assay for pyridoxine phosphate oxidase, FMN invariably produced a 10–30% stimulation of the measured activity of this enzyme, indicating that this enzyme is normally not saturated by its coenzyme in erythrocytes.

The pH-rate profiles of the enzymes for the conversion of pyridoxal, pyridoxine phosphate, and pyridoxine to PLP were also examined. The pH optima for pyri-

---

g/100 ml. The medium used for investigating the conversion of pyridoxine to PLP was modified so that the pH was 7.4 and the final hemoglobin concentration was 1.30 g/100 ml.
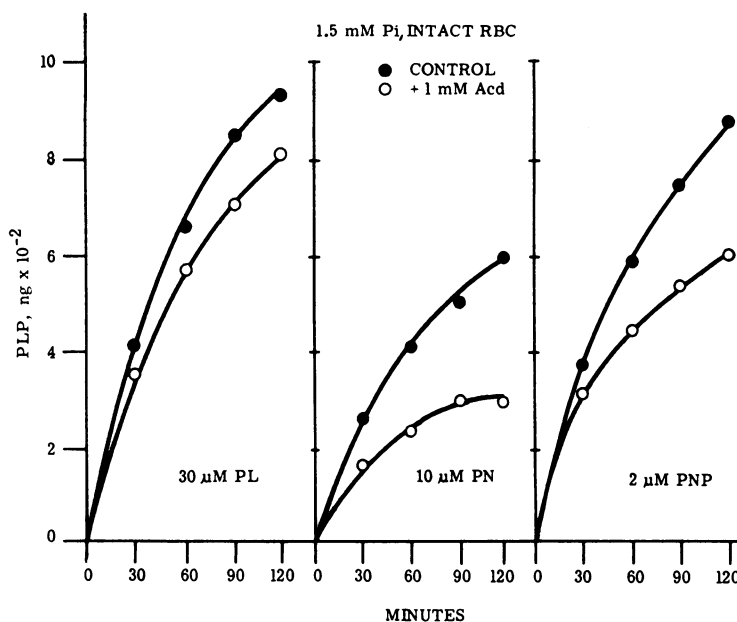
FIGURE 5 Effect of acetaldehyde (Acd) on the net formation of PLP by intact erythrocytes (RBC) in the presence of 1.5 mM Pi. Washed cells containing 185 mg hemoglobin were incubated in the 1.5 mM Pi medium as described for Fig. 3. Where indicated, 1 mM Acd was also present. Reactions were started by the addition of B_6 substrates: pyridoxal (PL), pyridoxine (PN), and pyridoxine phosphate (PNP). The final volumes were 10 ml, and incubation temperature was 32°C. Each point is the mean of duplicate determinations.

doxal kinase, pyridoxine phosphate oxidase, and the coupled reactions were 8.0, 7.4, and 8.0, respectively.

A previous report had postulated that pyridoxal serves as a regulator molecule of PLP metabolism in *Escherichia coli* since the pyridoxal kinase from this bacterial species is inactivated by pyridoxal on a time-dependent basis (19). Hence, experiments were performed to examine whether a similar control mechanism obtains in human erythrocytes. The supernatant fractions of red cell hemolysate were preincubated with pyridoxal, in concentrations as high as 1 mM and for durations as long as 1 h, in the presence of 0.5 mM

TABLE II

Erythrocyte Pyridoxal Kinase, and Pyridoxine Phosphate Oxidase Activities in Alcoholic and Non-alcoholic Subjects

	Number of subjects	Plasma PLP	Oxidase activity	Kinase activity
		ng/ml	*μg/h/g Hb*	*μg/h/g Hb*
Alcoholic	7	3.1–5.0	5.6–12.2	4.5–14.9
Nonalcoholic	8	8.2–25.1	3.0–7.9	3.7–12.4

The procedure was as described in Fig. 3, using the 80 mM Pi medium. The ranges of measured parameters are shown.

ZnSO$_4$ and 10 mM potassium phosphate, pH 6.0 (19). In no instance was inactivation of the kinase detected when it was assayed after the addition of ATP.

Erythrocytic pyridoxal kinase and pyridoxine phosphate oxidase activities of alcoholic subjects with low plasma PLP concentrations. The pyridoxal kinase and pyridoxine phosphate oxidase activities of intact erythrocytes from seven alcoholic subjects with abnormally low plasma PLP concentrations were compared with those from eight individuals from the control group (Table II). No significant differences were observed for either enzyme. Moreover, the total and specific activities of the two enzymes were comparable in magnitude in both normal and alcoholic subjects. These results are in contrast to those of Hines, reported in a preliminary communication, that the erythrocyte pyridoxal kinase activity was decreased in alcoholics during and up to 2 wk after alcohol administration (20).

Effect of ethanol and acetaldehyde on the net synthesis of PLP by intact human erythrocytes. The effects of ethanol and acetaldehyde on the net formation of PLP by human red cells were studied in the presence of either 1.5 mM Pi, a condition in which the activity of the B$_6$-phosphate phosphatase was preserved, or 80 mM Pi, where the activity of the erythrocytic phosphatase was inhibited. Ethanol, in concentrations as high as 70 mM, did not alter the net synthesis of PLP

by washed erythrocytes under either condition. In contrast, acetaldehyde ($\leq$1 mM) decreased the net conversion of pyridoxal, pyridoxine, and pyridoxine phosphate to PLP by erythrocytes incubated in 1.5 mM Pi (Fig. 5). After 2 h of incubation with 1 mM acetaldehyde, the amount of PLP synthesized from pyridoxine and pyridoxine phosphate was 30–50% less than that of the controls. Impairment of net PLP synthesis was less pronounced when pyridoxal was the substrate. The effect of varying the concentration of acetaldehyde on the net synthesis of PLP from pyridoxine is shown in Fig. 6. Significant impairment was evident even at concentrations as low as 0.05 mM. However, this effect of acetaldehyde was completely abolished when red cells were incubated in 80 mM Pi (Fig. 7). These results therefore imply that the action of acetaldehyde requires the presence of an active B_6-phosphate phosphatase.

Effect of acetaldehyde on PLP synthesis by the supernatant fraction of erythrocytic hemolysate. Since the hemolysate supernate of erythrocytes contains only the pyridoxal kinase and pyridoxine phosphate oxidase activities but no B_6-phosphate phosphatase activity, the effect of acetaldehyde on PLP synthesis by this fraction was examined. Acetaldehyde, 1 mM, did not affect the conversion of pyridoxal and pyridoxine phosphate to PLP by the hemolysate supernate (Fig. 8), thus further implicating the B_6-phosphate phosphatase as the
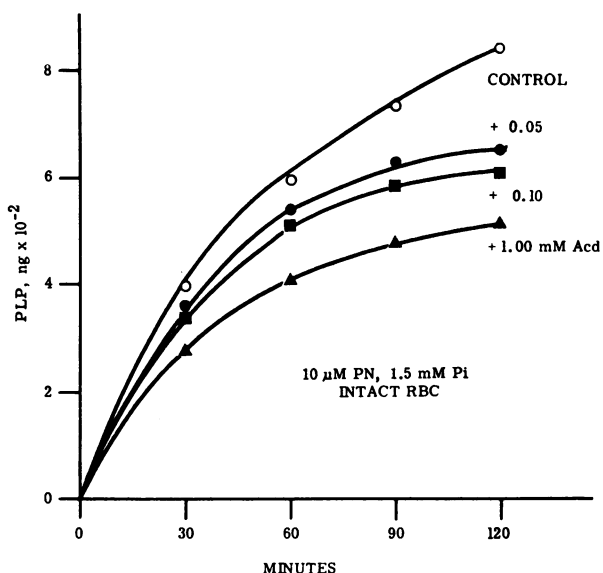

FIGURE 6 Effect of acetaldehyde (Acd) concentration on the net synthesis of PLP from pyridoxine (PN) by intact red cells (RBC) in media containing 1.5 mM Pi. Where indicated, 0.05, 0.10, or 1.00 mM Acd was added. Washed RBC containing 225 mg hemoglobin were used. Conditions were otherwise identical to Fig. 5. The values are means of duplicate assays.

mediator of the action of acetaldehyde on net PLP synthesis by intact erythrocytes.
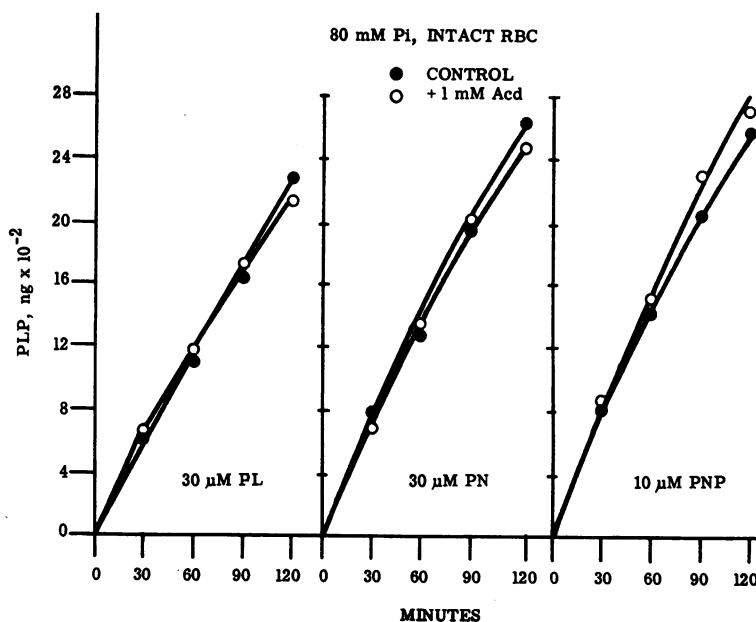

FIGURE 7 Net synthesis of PLP by intact erythrocytes (RBC) incubated in the 80 mM Pi medium, plus or minus 1 mM acetaldehyde (Acd). The 80 mM Pi medium was as described in Fig. 3. Washed RBC containing 188 mg hemoglobin were added. Reactions were initiated by addition of either pyridoxal (PL), pyridoxine (PN) or pyridoxine phosphate (PNP). Final volume, 10 ml; temperature, 32°C. Each value is the average of duplicate assays.
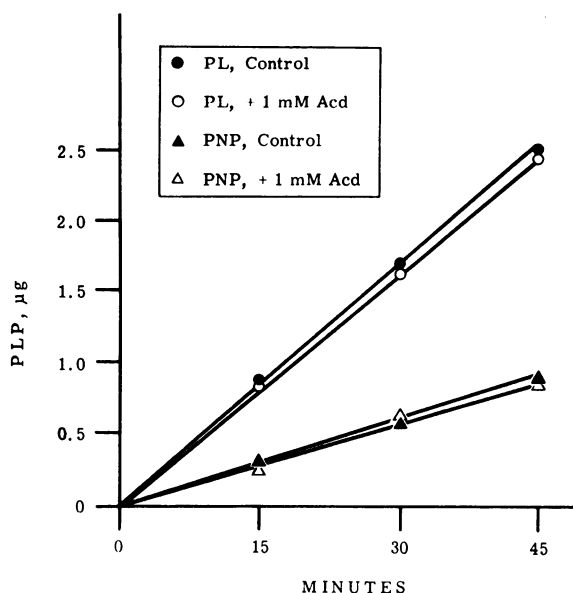
FIGURE 8 Effect of acetaldehyde (Acd) on the net formation of PLP by the supernatant fraction of hemolyzed human erythrocytes. Pyridoxal kinase activity was assayed in 0.1 M TEA (pH 8.0), 2.5 mM ATP, 1.0 mM $MgCl_2$, 20 μM pyridoxal (PL), and hemolysate supernate containing 170 mg hemoglobin. Pyridoxine phosphate oxidase activity was determined in 0.1 M TEA (pH 7.4), 30 μM pyridoxine phosphate (PNP) and hemolysate supernate containing 132 mg hemoglobin. Where indicated, 1 mM Acd was employed. Final volume, 10 ml; incubation temperature, 32°C.

Effect of acetaldehyde on PLP synthesis by a reconstituted system of hemolysate supernatant and sonicated membrane fractions. With the knowledge of the intracellular locations of pyridoxal kinase, pyridoxine phosphate oxidase, and the B_6-phosphate phosphatase, the entire PLP-metabolizing capacity of the erythrocyte can be restored by combining the erythrocyte membranes with the hemolysate supernate. Incubation of pyridoxine phosphate with the supernatant fraction in either 1.5 or 80 mM Pi, (Fig. 9 A) or with the combined mixture of supernate and sonicated ghosts in 80 mM Pi (Fig. 9 B) resulted in the same amount of PLP synthesized and the reactions were linear as a function of time. These results were expected since the supernatant fraction alone did not contain the B_6-phosphate phosphatase while phosphatase activity in the sonicated ghosts was inhibited by high concentrations of Pi. In the presence of 80 mM Pi, the addition of acetaldehyde (4 mM), with and without preincubation, resulted in little or no inhibitory effect on the net production of PLP by the recombined system of supernate plus sonicated ghosts (Fig. 9 B). In 1.5 mM Pi, the B_6-phosphate phosphatase activity of the sonicated ghosts was not inhibited and as a result, the net formation of PLP

from pyridoxine phosphate was drastically diminished (Fig. 9 C). Under these conditions, acetaldehyde, especially when preceded by preincubation, further curtailed the net formation of PLP. The action of acetaldehyde is thus apparent as an enhancement of the activity of the B_6-phosphate phosphatase.

DISCUSSION

Pyridoxal phosphate constitutes a substantial portion of the total vitamin B_6 compounds present in plasma and tissues and its concentration in plasma is considered to be a reliable indicator of the state of vitamin B_6 nutrition (21). It correlates directly with the PLP content of erythrocytes and appropriately with several functional criteria of the adequacy of vitamin B_6 nutrition, such as tryptophan load testing and the PLP-saturability of erythrocyte transaminases (22, 23). The relative constancy of plasma PLP values observed in this study in the six control subjects for durations as long as 6 mo further confirms the usefulness of this measurement for the assessment of vitamin B_6 nutritional status in
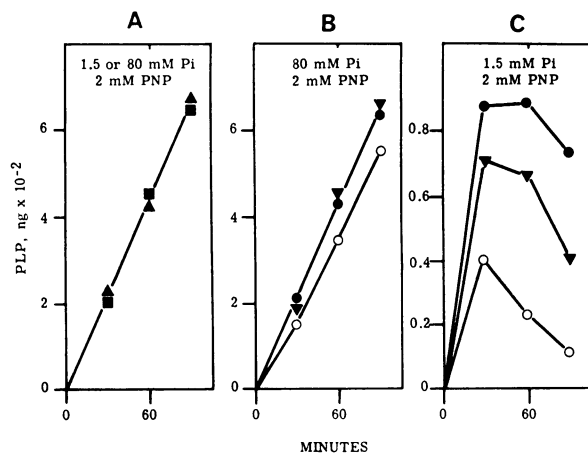

FIGURE 9 Effect of acetaldehyde (Acd) on the net synthesis of PLP of reconstituted broken cell preparations. The incubation media contained 10 mM TEA (pH 7.4), 1 mM $MgCl_2$, 80 mM potassium phosphate (pH 7.4) or 1.5 mM potassium phosphate (pH 7.4) plus 102 mM KCl. Where indicated, hemolysate supernate containing 170 mg hemoglobin, sonicated ghosts derived from 0.9 ml of packed erythrocytes, or 4 mM Acd was employed. Reactions were initiated by adding the substrate, 2 mM pyridoxine phosphate (PNP). When preincubation was done, it was carried out for 30 min in the absence of the substrate. (A) Supernatant fraction alone in either 1.5 or 80 mM Pi. ■——■, control; ▲——▲, plus Acd. (B) Supernatant fraction plus sonicated ghosts in 80 mM Pi. ●——●, control, with or without preincubation; ▼——▼, plus Acd; ○——○, with preincubation in the presence of Acd. (C) Supernatant fraction plus sonicated ghosts in 1.5 mM Pi. ●——●, control, with or without preincubation; ▼——▼, plus Acd; ○——○, plus Acd and preceded by preincubation.

man. The plasma levels for the control population in the present report are similar to those determined by Hamfelt (16), Chabner and Livingston (12), and Walsh (24), who used tyrosine apodecarboxylase and glutamic-oxaloacetic apotransaminase for assay, but are lower than those reported by Hines and Love (8) who employed apophosphorylase b. The decrease in the plasma PLP levels with age in the control population has also been observed by others (16, 24). Although no explanation is yet available for this phenomenon, it is interesting that this age-related decline in plasma PLP is preserved in the alcoholic population.

The present findings demonstrate that there is a high incidence of deranged PLP metabolism in chronic alcohol abuse and that it occurs before the appearance of liver disease. 53% of the alcoholic subjects had plasma PLP values less than 5 ng/ml, the lowest level encountered in the control group (Fig. 2). Since plasma PLP concentrations have been shown to correlate appropriately with other biochemical indicators of the state of vitamin B_6 nutrition, the data strongly suggest that a true state of PLP deficiency, albeit subclinical, prevails in many of the alcoholic patients.

The occurrence of altered vitamin B_6 metabolism in alcoholic individuals before the development of overt liver disease has important clinical implications. Pyridoxal phosphate functions as the essential coenzyme at numerous stages in the metabolism of proteins, carbohydrates, and fats and in the synthesis of many biologically important compounds, including biogenic amines and heme (25). Apart from the possibility that altered metabolism of PLP may have a causative role in the genesis of alcoholic liver disease (5–7), the repair of cellular damage from alcohol requires an increased need for nutrients; the failure to correct the disturbance in the metabolism of an essential vitamin may contribute to chronic tissue injury.

Hines has previously reported in preliminary communications that the pyridoxal kinase activities of erythrocytes and hepatic tissue are diminished in some alcoholic patients, and has suggested that this might be a cause of deranged PLP metabolism (10, 20). Our measurements of the pyridoxal kinase and pyridoxine phosphate oxidase activities in the erythrocytes of alcoholics with abnormally low plasma PLP levels (Table II) do not show impaired enzyme activities. It should be pointed out that Hines' subjects were chronic alcoholic volunteers who were ingesting the equivalent of a fifth of 86 proof whiskey per day and had developed abnormal liver function tests and sideroblastic changes in bone marrows at the time that the pyridoxal kinase activities were assayed. In contrast, the subjects in this study were chronic alcoholics devoid of hematologic or liver disease and who had abstained from alcohol for several days. Furthermore, Hines employed hemolyzed

cells for his assays whereas the assays in this study were performed with intact erythrocyte. The erythrocytes were incubated in the presence of 80 mM Pi, which precluded any interference by the phosphatase, and optimal concentrations of pyridoxal and pyridoxine phosphate were employed, thereby avoiding substrate inhibition (Fig. 4).

The studies with the intact erythrocytes demonstrate that the rate of net PLP synthesis and the cellular content of PLP are regulated not only by the activities of the PLP synthesizing enzymes, pyridoxal kinase, and pyridoxine phosphate oxidase, but also by a B_6-phosphate phosphatase (Figs. 3 and 9). The importance of the phosphatase as a regulator of PLP concentrations in tissues has not been fully appreciated in the past and considerations of regulatory mechanisms have centered primarily on modes of inhibition or inactivation of the kinase enzyme (19, 26). The control mechanism based on pyridoxal as a regulatory molecule (19) probably does not apply to man since our findings indicated that pyridoxal does not inactivate the pyridoxal kinase of erythrocytes. The action of the B_6-phosphate phosphatase is relevant to red cell metabolism since high concentrations of PLP have been shown to interfere with the activities of many glycolytic and pentose phosphate shunt enzymes (27). Anderson and associates have also suggested that the phosphatase enzyme in erythrocytes may play an important role in converting pyridoxine to pyridoxal which is then transported from erythrocytes to other tissues (17).

The pronounced influence of Pi on the net amount of PLP formed in intact cells can be explained by its inhibitory action upon B_6-phosphate phosphatase (Fig. 3), an effect which was reproduced in the reconstituted system of hemolysate supernate and sonicated membranes (Fig. 9). In the intact cell, high concentrations of Pi in the medium will also accelerate the glycolytic pathway (28) and the transport of vitamin B_6 compounds (18). These effects, however, do not obtain in the reconstituted system.

The oxidation product of alcohol, acetaldehyde, decreased the net synthesis of PLP in intact human red cells in vitro (Figs. 5 and 6). The experiments using intact cells and broken cell preparations show clearly that this phenomenon is the result of an accelerated degradation of the phosphorylated B_6 compounds, mediated by the activity of the neutral B_6-phosphate phosphatase (Figs. 6, 7, and 9). Since ethanol itself had no demonstrable effect, the observed phenomenon and mechanism therefore represent a unique action of acetaldehyde.

The molecular basis for the apparent enhancement of the activity of the B_6-phosphate phosphatase is unknown. Acetaldehyde may alter the activity of this enzyme directly by interaction with amino groups

through Schiff base formation or indirectly through peroxidation of the lipids of the plasma membrane. We have observed that acetaldehyde will also alter the activity of other membrane associated enzymes in erythrocytes: at 0.05 mM, acetaldehyde effectively inhibited the activities of acetylcholinesterase and p-nitrophenylphosphate phosphatase of erythrocytic membrane preparations.[2] An alternative hypothesis for the enhancement of PLP hydrolysis is that acetaldehyde competes with PLP for binding to proteins in the erythrocytes and displaces bound PLP. Preliminary studies indicate that while free PLP is readily hydrolyzed by the membrane-associated B_6-phosphate phosphatase, PLP bound to intracellular proteins of the erythrocyte or to bovine serum albumin is not.[3] Studies to delineate these possible mechanisms of acetaldehyde action are in progress.

Whether or not the effect of acetaldehyde as here shown with human red cells plays a significant role in the genesis of deranged PLP metabolism in chronic alcohol abuse cannot be ascertained at this time for several reasons. There is presently little information regarding the importance of the B_6-phosphate phosphatase in the control of intracellular PLP levels of tissues other than in the erythrocyte (17, 29). Furthermore, little is known of the role of the different organs in the interconversion of B_6 compounds in vivo (30–32). Human red cells were used in these studies as a first model because of their easy accessibility and the presence of easily measurable PLP-synthesizing and degradative enzyme activities. Similar studies need to be performed with cells from other sources, particularly those possessing high PLP synthesizing capacities (e.g., liver, kidney, spleen, and brain). In this regard, the liver is of particular interest, since this organ appears to be the principal source of circulating PLP[4] which presumably is then transferred to tissues with low PLP synthesizing capacity, e.g. skeletal muscle. Moreover, since the liver is the major site of ethanol oxidation, it appears that the concentration of acetaldehyde in situ may be higher in this tissue than in other organs. Anderson, Fulford-Jones, Child, Beard, and Bateman (17) have recently suggested that the role of erythrocytes in vitamin B_6 metabolism in vivo is to convert pyridoxine to pyridoxal. They speculate that pyridoxal may be the physiologically important vitamer, more readily utilized by the cells of other tissues. However, such a consideration would pertain only if pyridoxal is preferentially transported or if pyridoxine phosphate oxidase activity is low or absent in other tissues. These aspects of vitamin B_6 metabolism also remain to be explored.

[2] Scott, P., L. Lumeng, and T. K. Li. Unpublished data.
[3] Lumeng, L., and T. K. Li. Unpublished data.
[4] Lumeng, L., R. E. Brashear, and T. K. Li. Manuscript in preparation.

Although the majority of the metabolic consequences of alcohol are considered to be mediated by its effect on the redox state of the nicotinamide adenine dinucleotide coenzyme in liver (33), several biochemical actions of ethanol have now been ascribed to acetaldehyde (34–39). However, there is, unfortunately, still some uncertainty with regard to the concentration of acetaldehyde attainable in circulation and in tissues during the imbibition of alcoholic beverages. Blood values following alcohol ingestion have been reported to range from 1.0 to 7.0 $\mu g/ml$ (0.023–0.160 mM) as determined by a variety of methods (34). More recent studies by Truitt (40) and by Majchrowicz and Mendelson (41) indicate that the concentration of "free" acetaldehyde in blood is usually less than 1.0 $\mu g/ml$ during the consumption of alcoholic solutions low in or devoid of congeners and 1.1–1.5 $\mu g/ml$ when beverages high in congener content are given. However, there is also an indeterminate quantity of acetaldehyde in erythrocytes and, perhaps, other tissues which is chemically bound and which is released by protein precipitants (42).

The range of acetaldehyde concentrations employed in these studies is comparable to those employed to demonstrate an inhibitory effect of acetaldehyde on brain metabolism (36–38, 43), but are much lower than those used to discern the metabolic effects of acetaldehyde on cardiac and hepatic tissues (38, 39). A significant decrease in the net synthesis of PLP of human erythrocytes was evident at concentrations of acetaldehyde as low as 0.05 mM (Fig. 6). This concentration of total acetaldehyde added to incubations in vitro is of the same order of magnitude as the "free" acetaldehyde blood levels reported by Truitt (40) and Majchrowicz and Mendelson (41) in their human studies in vivo. Such quantitative comparisons are noteworthy in evaluating the pathophysiologic significance of the metabolic effects of acetaldehyde.

Multiple vitamin deficiencies are frequently encountered in chronic alcohol abuse. Folate and vitamin B_6 deficiencies as etiologic factors in the anemia of alcoholism have been well characterized (44). In addition to primary vitamin deficiency due to inadequate intake, ethanol per se or its metabolic products may produce conditioned vitamin deficiency states (45) due to alterations in one or more of several processes which affect vitamin metabolism; namely, absorption, conversion, storage, tissue utilization, and excretion. Eichner and Hillman (46) recently presented evidence that alcohol may interfere with either the storage of folate or the conversion of folic acid to N-5-methyltetrahydrofolic acid. The studies of Hines and Cowan have indicated that during alcohol ingestion, the net formation in vivo of PLP from pyridoxine is decreased (4). The in vitro studies reported here suggest that this action of alcohol

may be mediated by acetaldehyde through an acceleration of the hydrolysis of phosphorylated B_6 compounds.

REFERENCES

1. Rodwell, V. W. 1970. Metabolism of vitamin B_6. *In* Metabolic Pathways. D. M. Greenberg, editor. Academic Press, Inc., New York. 3rd edition. **4**: 411.
2. Leevy, C. M., E. Valdellon, and F. Smith. 1971. Nutritional factors in alcoholism and its complications. *In* Biological Basis of Alcoholism. Y. Israel and J. Mardones, editors. Wiley-Interscience, New York. 365.
3. Lerner, A. M., L. M. DeCarli, and C. S. Davidson. 1958. Association of pyridoxine deficiency and convulsions in alcoholics. *Proc. Soc. Exp. Biol. Med.* **98**: 841.
4. Hines, J. D., and D. H. Cowan. 1970. Studies on the pathogenesis of alcohol-induced sideroblastic bone-marrow abnormalities. *N. Engl. J. Med.* **283**: 441.
5. French, S. W., and J. Castagna. 1967. Some effects of chronic ethanol feeding on vitamin B_6 deficiency in the rat. *Lab. Invest.* **16**: 526.
6. Okada, M., and A. Ochi. 1971. The effect of dietary protein level on transaminase activities and fat deposition in vitamin B_6-depleted rat liver. *J. Biochem. (Tokyo).* **70**: 581.
7. Greenberg, L. D. 1964. Arteriosclerotic, dental, and hepatic lesions in pyridoxine-deficient monkeys. *Vitam. Horm.* **22**: 677.
8. Hines, J. D., and D. S. Love. 1969. Determination of serum and blood pyridoxal phosphate concentrations with purified rabbit skeletal muscle apophosphorylase b. *J. Lab. Clin. Med.* **73**: 343.
9. Frank, O., A. Luisada-Opper, M. F. Sorrell, A. D. Thomson, and H. Baker. 1971. Vitamin deficits in severe alcoholic fatty liver of man calculated from multiple reference units. *Exp. Mol. Pathol.* **15**: 191.
10. Hines, J. D. 1970. Correspondence. *N. Eng. J. Med.* **283**: 1173.
11. Criteria Committee, National Council on Alcoholism. 1972. Criteria for the diagnosis of alcoholism. *Ann. Intern. Med.* **77**: 249.
12. Chabner, B., and D. Livingston. 1970. A simple enzymic assay for pyridoxal phosphate. *Anal. Biochem.* **34**: 413.
13. Tamura, Z., and S. Takanashi. 1970. Fluorometric determination of pyridoxal and pyridoxal 5'-phosphate in biological materials by the reaction with cyanide. *Methods Enzymol.* **18**: 471.
14. Beutler, E. 1971. Red Cell Metabolism—A Manual of Biochemical Methods. Grune and Stratton, New York. 13.
15. Peterson, E. A., and H. A. Sober. 1954. Preparation of crystalline phosphorylated derivatives of vitamin B_6. *J. Am. Chem. Soc.* **76**: 169.
16. Hamfelt, A. Age variation of vitamin B_6 metabolism in man. *Clin. Chim. Acta.* **10**: 48.
17. Anderson, B. B., C. E. Fulford-Jones, J. A. Child, M. E. J. Beard, and C. J. T. Bateman. 1971. Conversion of vitamin B_6 compounds to active forms in the red blood cell. *J. Clin. Invest.* **50**: 1901.
18. Yamada, K., and M. Tsuji. 1968. Transport of vitamin B_6 in human erythrocytes. *J. Vitaminol. (Osaka).* **14**: 282.
19. White, R. S., and W. B. Dempsey. 1970. Purification and properties of vitamin B_6 kinase from *Escherichia coli B. Biochemistry.* **9**: 4057.
20. Hines, J. D. 1969. Altered phosphorylation of vitamin B_6 in alcoholic patients induced by oral administration of alcohol. *J. Lab. Clin. Med.* **74**: 882. (Abstr.)
21. Sauberlich, H. E., J. E. Canham, E. M. Baker, N. Raica, Jr., and Y. F. Herman. 1972. Biochemical assessment of the nutritional status of vitamin B_6 in the human. *Am. J. Clin. Nutr.* **25**: 629.
22. Hamfelt, A. 1967. Enzymatic determination of pyridoxal phosphate in plasma by decarboxylation of L-tyrosine-^{14}C (U) and a comparison with the tryptophan load test. *Scand. J. Clin. Lab. Invest.* **20**: 1.
23. Hamfelt, A. 1967. Pyridoxal phosphate concentration and aminotransferase activity in human blood cells. *Clin. Chim. Acta.* **16**: 19.
24. Walsh, M. P. 1966. Determination of plasma pyridoxal phosphate with wheat germ glutamic-aspartic apotransaminase. *Am. J. Clin. Pathol.* **46**: 282.
25. Sauberlich, H. E. 1968. Biochemical systems and biochemical detection of deficiency. *In* The Vitamins. W. H. Sebrell, Jr., and R. E. Harris. editors. Academic Press, Inc., New York. 2nd edition. **2**: 44.
26. Ebadi, M. S., E. E. McCoy, and R. B. Kugel. 1970. Interrelationships between pyridoxal phosphate and pyridoxal kinase in rabbit brain. *J. Neurochem.* **17**: 941.
27. Srivastava, S. K., and E. Beutler. 1972. The effect of normal red cell constituents on the activities of red cell enzymes. *Arch. Biochem. Biophys.* **148**: 249.
28. Rose, I. A., and J. V. B. Warms. 1966. Control of glycolysis in the human red blood cells. *J. Biol. Chem.* **241**: 4848.
29. Turner, J. M., and F. C. Happold. 1961. Pyridoxamine phosphate-oxidase and pyridoxal phosphate-phosphatase activities in *Escherichia coli. Biochem. J.* **78**: 364.
30. Johansson, S., S. Lindstedt, and H. G. Tiselius. 1968. Metabolism of [$^3H_8$] pyridoxine in mice. *Biochemistry.* **7**: 2327.
31. Colombini, C. E., and E. E. McCoy. 1970. Vitamin B_6 metabolism. The utilization of [^{14}C] pyridoxine by the normal mouse. *Biochemistry.* **9**: 533.
32. Snell, E. E., and B. E. Haskell. 1971. The metabolism of vitamin B_6. *Compr. Biochem.* **21**: 47.
33. Hawkins, R. D., and H. Kalant. 1972. The metabolism of ethanol and its metabolic effects. *Pharmacol. Rev.* **24**: 67.
34. Truitt, E. B., Jr., and M. J. Walsh. 1971. The role of acetaldehyde in the actions of ethanol. *In* The Biology of Alcoholism. B. Kissin and H. Begleiter, editors. Plenum Press, New York. **1**: 161.
35. Duritz, G., and Truitt, E. B., Jr. 1966. Importance of acetaldehyde in the action of ethanol on brain norepinephrine and 5-hydroxy-tryptamine. *Biochem. Pharmacol.* **15**: 711.
36. Lahti, R. A., and E. Majchrowicz. 1969. Acetaldehyde—

An inhibitor of the enzymatic oxidation of 5-hydroxy-indoleacetaldehyde. *Biochem. Pharmacol.* **18**: 535.

37. Kiessling, K. H. 1962. The effect of acetaldehyde on rat brain mitochondria and its occurrence in brain after alcohol injection. *Exp. Cell Res.* **26**: 432.

38. Ammon, H. P. T., C. J. Estler, and F. Heim. 1969. Inactivation of coenzyme A by ethanol-I. Acetaldehyde as mediator of the inactivation of coenzyme A following the administration of ethanol *in vivo*. *Biochem. Pharmacol.* **18**: 29.

39. Schreiber, S. S., K. Briden, M. Oratz, and M. A. Rothschild. 1972. Ethanol, acetaldehyde, and myocardial protein synthesis. *J. Clin. Invest.* **51**: 2820.

40. Truitt, E. B., Jr. 1971. Blood acetaldehyde levels after alcohol consumption by alcoholic and nonalcoholic subjects. *In* Advances in Mental Science–Biological Aspects of Alcohol. M. K. Roach, W. M. McIssac, and P. J. Creaven, editors. University of Texas Press, Austin. **3**: 212.

41. Majchrowicz, E., and J. H. Mendelson. 1970. Blood concentrations of acetaldehyde and ethanol in chronic alcoholics. *Science (Wash. D. C.).* **168**: 1100.

42. Truitt, E. B., Jr. 1970. Ethanol-induced release of acetaldehyde from blood and its effect on the determination of acetaldehyde. *Quart. J. Stud. Alcohol.* **31**: 1.

43. Lahti, R. A., and E. Majchrowicz. 1967. The effects of acetaldehyde on serotonin metabolism. *Life Sci.* **6**: 1399.

44. Eichner, E. R., and R. S. Hillman. 1971. The evolution of anemia in alcoholic patients. *Am. J. Med.* **50**: 218.

45. Ershoff, B. H. 1948. Conditioning factors in nutritional disease. *Physiol. Rev.* **28**: 107.

46. Eichner, E. R., and R. S. Hillman. 1973. Effect of alcohol on serum folate level. *J. Clin. Invest.* **52**: 584.