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DO GLUCOCORTICOIDS AFFECT VITAMIN B6 METABOLISM IN CELLS IN WHICH THE STEROID INDUCES B6 DEPENDENT ENZYMES?

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Shawn Tab Sussmane

December 1993

ACKNOWLEDGEMENTS

I would like to thank my major professor, Dr. John Koontz, for his guidance, training, and willingness to help. I would like to thank Dr. Wesley Wicks for his comments and assistance. Also, I would like to thank Dr. Jorge Churchich for generously providing pure pyridoxal kinase and for the assistance in making the pyridoxal kinase work possible.

ABSTRACT

Two observations in the literature have led us to believe that glucocorticoids may affect the metabolism of the vitamers of pyridoxal-5-phosphate (PLP). The steroid induces many PLP dependent enzymes in the liver and it has been suggested that PLP regulates steroid hormone action.

We have examined the effect of the synthetic glucocorticoid, dexamethasone, on vitamin B6 metabolism in a rat hepatoma cell line, KRC-7. Previous experiments, which are verified herein, have demonstrated that this cell line elicits a typical sensitivity to dexamethasone. Pyridoxal kinase and pyridoxine-5-phospate or pyridoxamine-5-phospate oxidase (the two most important enzymes in the biosynthesis of the physiologically active form of the vitamer PLP) were measured in cells following treatment with dexamethasone. The level of 4-pyridoxic acid in the culture medium was assayed as a measure of the rate of catabolism of the vitamers. The activities of pyridoxal kinase, PNP-oxidase, or the formation of 4-pyridoxic acid do not change after cells are treated with 10^{-6}M dexamethasone for a period of 24 hours. These results indicate that the glucocorticoid does not alter the metabolism of the vitamers.

A novel fluorometric assay was proposed to measure pyridoxal kinase activity, an enzyme which phosphorylates pyridoxal to form pyridoxal-5-phospate. The basis of this assay relies on th reaction of hydroxylamine with the aldehyde groups of both pyridoxal (PL) and pyridoxal-5-phosphate (PLP) to form PL-oxime and PLP-oxime. Addition of hydroxylamine to either PL or PLP increases the fluorescence at 450 nm upon excitation at 380 nm. Reaction kinetics indicate that PLP reacts much faster with

hydroxylamine than does PL. Therefore, the fluorescence due to the PLP-oxime can easily be distinguished from the fluorescence due to the PL-oxime. We demonstrate that this assay satisfies the requirements for being both time and concentration dependent using both purified pyridoxal kinase as well as enzyme activity detected in crude extracts of cultured hepatoma cells.

Separately, I have utilized culture conditions which have been reported to deplete the intracellular stores of PLP. One would predict that under these constrained conditions the cells would be more acutely sensitive to the potential effects of dexamethasone on vitamer metabolism. I measured not only pyridoxal kinase, the rate limiting enzyme in PLP biosynthesis, but also the holoenzyme to total enzyme ratios of two PLP dependent enzymes, aspartate aminotransferase and tyrosine aminotransferase. This gives an indication of whether these culture conditions have lead to depletion of PLP in the cells. Finally, I also measured the induction of tyrosine aminotransferase enzyme activity by dexamethasone under these conditions.

Under the initial constraints imposed upon the cell, i.e. treatment of non-growing cells with 5mM 4-deoxypyridoxine or incubation of non-growing cells in pyridoxal deficient medium, there was no effect on basal TAT activity, TAT holoenzyme to total enzyme ratio, or TAT fold induction by dexamethasone. Basal AAT activity did not change nor did its holoenzyme to total enzyme ratio.

These initial results differed from previously published reports. Therefore, I carried out the same experiments using the conditions designed to deplete PLP using growing cells. Under these circumstances, the basal TAT activity increases, the TAT

holoenzyme to total enzyme ratio decreases and TAT induction by dexamethasone does not change. AAT basal activity decreases and the holo to total enzyme ratios decrease. The changes in basal TAT activity, the holo/total enzyme activity and the loss in basal AAT corroborate previous reports. The decrease in the holo/total activity ratios for both TAT and AAT and the loss of basal AAT indicate that the cells are being depleted of intracellular PLP under these conditions. Thus, while attempts to deplete PLP in non-growing, resting cells were unsuccessful, it was readily accomplished in growing, dividing cells.

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INTRODUCTION

The form of vitamin B6 that is usually taken in by eucaryotes is either pyridoxal (PL) or pyridoxine (PN). Buss et al. reports that using [^{3}H]PN to measure uptake in the perfused small intestine of the rat, PN is absorbed by passive diffusion (1). Using [^{3}H]PL Mehanso et al. reports that PL is absorbed by passive diffusion in the perfused small intestine of the rat (2). The physiologically active form of vitamin B6 is pyridoxal-5-phosphate (PLP) (3).

The biosynthesis of PLP can occur as shown in Figure 1 (4). Phosphorylation of PL by the kinase forms PLP directly. That same kinase utilizes PN, or pyridoxamine (PM) resulting in the formation of their respective phosphorylated forms, pyridoxine-5-phosphate (PNP) or pyridoxamine-5-phosphate (PMP). An oxidase which uses either PNP or PMP produces PLP (4). 4-pyridoxic acid, which is secreted as a waste product from the cell, is formed from the irreversible breakdown of PL (4).

PLP is a required cofactor for many enzymatic reactions including aminotransferases. Tyrosine aminotransferase (TAT) and aspartate aminotransferase (AAT) utilize PLP to form a Schiff base with the amino acid to transfer the amino group to the keto acid. The Km of AAT for PLP has been reported to be 1 nM (5) and the Km of TAT for PLP has been reported to be 360 nM (6). Aspartate aminotransferase has a very low Km for PLP, indicating a very tight association between cofactor and enzyme. Lipson et al. reported that in human skin fibroblast line 86 grown in PL deficient medium for four passages AAT activity decreases by 50 percent (7). This decrease in AAT activity was accompanied by a decrease in the holo/total

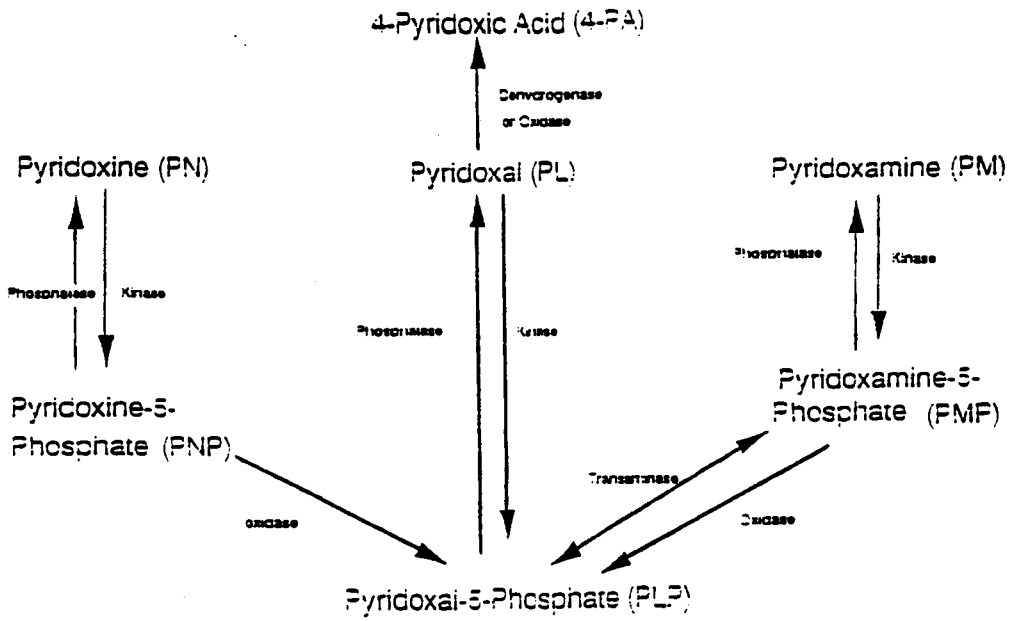

Figure 1. Interconversion of the B6 vitamers.

enzyme ratio from 100% to 70% after three passages in PL deficient media (7). Subsequently, Shibuya et al. later reported a decrease in the activity and amount of AAT in the liver using male Wistar strain rats that were maintained on a pyridoxine deficient diet for 4 weeks (8). The high affinity of AAT for PLP and the decrease in the amount of AAT produced in PL deficient conditions indicate an instability of the apoenzyme form of AAT.

The interaction of PLP with PLP dependent proteins is reversible. Thus, when two proteins which bind PLP are incubated in limiting concentrations of PLP, the enzyme with the higher affinity is more saturated. Thus, Churchich has reported that from a starting mixture of apo AAT (Km for PLP= 1 nM) and holo cystathionase (Km for PLP=140 nM), increasing AAT activity is obtained with time of incubation (9). Masugi et al. reports PLP can bind to bovine serum albumin to form a complex which is difficult to dissociate even under intensive dialysis (10). In addition Masugi et al. reports that incubation of apo AAT with a bovine serum albumin:PLP complex, results in a time dependent accumulation of active AAT. These results indicate that AAT can remove PLP bound to bovine serum albumin and also from holo cystathionase. PLP can also bind to human serum albumin (HSA), and this complex has been suggested to be a storage and transport molecule for PLP (11). Since cofactor transfer can occur, it would seem that in times of B6 depletion enzymes with a higher affinity for cofactor would retain their cofactor more than enzymes with a lower affinity e.g. PLP could be transfered from TAT to AAT.

Lee et al. reported a decrease in the synthesis of TAT in pyridoxine deficient rat liver (12). Disorbo and Litwack reported an increase in the fold induction of TAT by dexamethasone when the cells were grown in pyridoxal deficient media (13).

Glucocorticoids diffuse across the plasma membrane and bind to the cytoplasmic glucocorticoid receptor. Upon hormone binding to the receptor there is a release of two heat shock protein molecules and a p59 protein. The hormone receptor complex forms a homodimer and enters the nucleus where it binds to DNA and affects the transcriptional machinery to regulate transcription (14).

Dexamethasone, a synthetic glucocorticoid, has been shown to induce an increase in the amount of many PLP dependent enzymes by increasing the transcription of their mRNAs (15). Among the enzymes induced are: TAT (15), AAT (16), ornithine aminotransferase (17), alanine aminotransferase (18), and L-serine dehydratase (19).

Allgood and Cidlowski report that excessive pyridoxal supplementation in the media decreases the activity of the mouse mammory tumor virus promoter cotransfected into cells with the human glucocorticoid receptor expression vector (20). In addition, incubation of cells with 4-deoxypyridoxine (4-dPN), a competitive inhibitor of PL kinase (20), enhances the activity of a glucocorticoid responsive promoter cotransfected into cells with the human glucocorticoid receptor expression vector (20).

Maksymowych et al. report that omitting pyridoxine from the medium of the rat hepatoma FAZA cells increased the induction of TAT activity by glucocorticoids implying that PLP modulates steroid hormone action (21). Maksymowych et al. also report that in vitro PLP inhibits the binding of the activated glucocorticoid receptor to

DNA-cellulose suggesting a direct interaction of PLP with the glucocorticoid receptor (21). The above results suggest that intracellular PLP regulates glucocorticoid receptor mediated gene expression by interfering with the receptor binding to DNA.

In summary, four lines of evidence lead one to hypothesize an effect of glucocorticoids on vitamin B6 metabolism. Depletion of intracellular PLP allows greater gene induction by glucocorticoids. Incubation of cells with excess PL decreases glucocorticoid induced gene expression. PLP apparently inhibits in vitro receptor DNA interactions. Glucocorticoids induce the gene expression of a substantial number of PLP dependent proteins. Thus, it is logical to hypothesize that the steroid would cause an increase in the amount of intracellular PLP to accomodate the increased demand for the cofactor and that this increased level of cofactor could act as a feedback control on receptor activity.

I addressed this hypothesis in several ways. I investigated the effect of the steroid on PNP-oxidase and PL kinase, the two major enzymes controlling PLP generation. I also examined the steroid effect on the extracellular accumulation of 4-pyridoxic acid, the excreted end product of vitamer breakdown. In addition, I performed similar measurements in cells manipulated to deplete the intracellular pools of PLP. In these experiments I also measured AAT and TAT basal activities, holo/total activity ratio, and inducibility of TAT by glucocorticoids.

In experiments to measure the PL-kinase and PNP-oxidase in extracts prepared from rat hepatoma cells, we initially attempted to use the phenylhydrazine assay but detected no activity. Current methods for assaying pyridoxal kinase include a

radiometric assay (23), the phenylhydrazine assay (24), and a direct colorimetric assay (25). The radiometric assay is a very sensitive assay which utilizes tritiated pyridoxine as the substrate. The phenylhydrazine assay is a colorimetric assay that is based on phenylhydrazine reacting with both pyridoxal and pyridoxal-5-phosphate to form a hydrazone complex which yields a yellow color absorbing maximally at 410 nm. The direct colorimetric assay takes advantage of the difference in absorptivity of PLP which absorbs at 388 nm compared with PL which does not absorb at 388 nm. The assay follows the increase in absorption at 388 nm during a continuous assay. To avoid the use of radioisotopes we have developed a fluorometric assay that is more sensitive than the phenylhydrazine or continuous colorimetric methods. Beeler and Churchich reported that aminooxy derivatives react with pyridoxal-5-phosphate and pyridoxal to produce a fluorescent oxime (22). Hydroxylamine is one of a group of many aminooxy derivatives which react with PLP as well as PL. We present experiments to demonstrate the suitability of this assay for pyridoxal kinase.

PART I.

ENZYME STUDIES

MATERIALS AND METHODS

Cell Culture

KRC-7 rat hepatoma cells were grown in monolayer culture in Dulbecco's modified Eagle's medium containing 7.5% v/v iron-supplemented calf serum (Hyclone), penicillin (50 units/ml), and streptomycin (0.1 mg/ml), at 37^0 C in a humidified atmosphere of 90% air, 10% CO_2. Dulbecco's modified Eagle's medium without pyridoxal was prepared from individual components.

Elimination of Vitamin B6 from Calf Serum

The method of Lipson et al. (7) was used to deplete the PL from the serum. Briefly, calf serum was dialyzed for 24 hrs at 4^0 C against one change of a solution of 5 mM hydroxylamine in phosphate buffered saline (PBS) (10 mM $NaKHPO_4$ at pH 7.4, 130 mM NaCl, 5 mM KCl). The serum was then dialyzed against two changes of a PBS solution without hydroxylamine.

Experimental Design

The cells are depleted of PLP by two different methods. The cells are incubated in pyridoxine deficient media (PL-); or, they are incubated in media containing 4-dPN. The precursor to PLP used in cell culture media is pyridoxine or pyridoxal. It has been reported that a decrease in intracellular PLP levels results after cells are grown in pyridoxine deficient media (13). Cells were grown in 3 types of serum containing medium: 1) with pyridoxal (i.e. normal media PL+), 2) without pyridoxal (PL-), and

3) pyridoxal plus the addition of 4-deoxypyridoxine (4-dPN). For each of the three conditions there are dex treated samples and untreated controls. Experiments were performed using growing and non-growing cells. Non-growing cells are confluent cells maintained viable over the 8 day time period. Growing cells were maintained viable in a nonconfluent status. The 2 and 4 day cells were subcultured once in the appropriate media and the 6 and 8 days were subcultured twice in the appropriate media to prevent them from becoming confluent.

The transaminases studied are assayed for holoenzyme, which is the enzyme activity measured without an exogenous supply of cofactor, and total enzyme, which is the enzyme activity measured with an exogenous supply of cofactor. 4-dPN was dissolved in PBS and added to a final concentration of 5 mM. Dexamethasone was dissolved in PBS and added, at indicated times, to a final concentration of 1 uM. Cells were transferred to a serum-free medium for a period of 24 hours and then treated with dex for an additional 24 hours. Fold induction is defined as enzyme specific activity from dex treated cells divided by the enzyme specific activity of control, untreated cells.

Preparation of Cell Extracts

At the indicated times the media was aspirated and the cells were washed with ice cold PBS. The cells were collected in PBS and transferred to a microfuge tube. Cells were lysed by adding NP-40 to a final concentration of 0.1 % v/v and mixing. The solution was centrifuged for 15 min at 15,000 rpm and the supernatant was assayed for

enzyme activity. Experiments were performed to demonstrate that 0.1% NP-40 did not interfere with the enzyme assays for any of the enzymes discussed below.

Enzyme Assays

Tyrosine aminotransferase was assayed by the method of Diamondstone (26).

Aspartate aminotransferase was assayed by the method of Karmen (27).

Pyridoxal kinase (PK) and PNP oxidase were measured using fluorescent methods. The pyridoxal kinase substrate solution described in (28) is a 0.1 M potassium phosphate solution at pH 6.4 containing : 0.2 mM pyridoxal, 1 mM ATP, and 0.1 mM zinc. The PNP oxidase substrate solution is a 0.1 M potassium phosphate solution at pH 9 containing 0.1 mM pyridoxine-5-phosphate. Pyridoxine -5-phosphate was prepared by reacting sodium borohydride with PLP (29). Protein concentrations were determined by the method of Bradford (30).

Determination of 4-pyridoxic acid

The cells were grown and treated with dex as described above. The media was collected from untreated control and dex-treated cultures after 24 hours of dex treatment. One ml of trichloroacetic acid (10% w/v) is added per ml of media. The mixture is heated at 50^0 C for 20 minutes and centrifuged at 4,000 rpm. The supernatant is extracted twice with equal volumes of diethylether (peroxide free). The aqueous phase is used for further study.

The separation of 4-pyridoxic acid was accomplished by the method of Contractor and Shane (31). Briefly, a phosphocellulose column was prepared and equilibrated with 0.01 M acetic acid. Following application of the ether-extracted sample to the column (1 or 2 ml), 4-pyridoxic acid is eluted in the first fractions when washing with 0.01 M acetic acid (10 ml). 4-Pyridoxic acid fluoresces at 430 nm with an excitation wavelength of 340 nm. Using a Perkin Elmer LKB50, fluorescence of the samples was measured to detect pyridoxic acid. The fractions which fluoresce were lyophilized and resuspended in 50 ul of water. To control for the recovery of 4-pyridoxic acid, the recovery of phenol red, which is a dye indicator present in the medium, was measured by its absorption at 558 nm. This is appropriate because the concentration of phenol red in all of the media is uniform and the recovery of 4-pyridoxic acid can be compared to the recovery of phenol red. The concentration of 4-pyridoxic acid was determined by recording the fluorescence at 430 nm upon exciting at 340 nm of known concentrations of 4-pyridoxic acid. To guarantee that I was measuring material secreted into the media by the cells I checked the fresh media for 4-pyridoxic acid and could detect none.

Thin layer chromatography was performed to verify that the fluorescence migrated with authentic pyridoxic acid. The developing solvent consists of propanol, ammonium hydroxide, and water in a ratio of 34:47:10. A Polygram Cel 300 microcrystaline plate was used. A known standard of pyridoxic acid was run along side the eluted samples.

RESULTS

Enzyme Studies from Non-growing Cells

The two enzymes involved in the formation of PLP which we measured are pyridoxal kinase and PNP oxidase. These activities were measured in extracts isolated from control, untreated cells and in extracts from cells incubated with the synthetic glucocorticoid, dexamethasone. Neither pyridoxal kinase nor PNP oxidase activities change detectably in response to the glucocorticoid (Figure 2).

Both of these activities are critical for the formation of the biologically active form of the cofactor from precursors. That their specific activity is unchanged in dex treated cells implies no effect on PLP biosynthesis. As a further test of whether the steroid might influence the metabolism of the vitamers, we measured the content of 4-pyridoxic acid in the medium. Assuming the steroid mediates an accumulation of PLP as it induces PLP-requiring enzymes, and since the rate limiting enzyme in PLP synthesis is unaffected by dexamethasone, the possible accumulation of PLP might be mediated by a decrease in the catabolism of the vitamer, which would be reflected in a decrease in the secretion of the major breakdown product of the vitamers.

Results from these studies demonstrated that the steroid had no effect on the release of 4-pyridoxic acid into the medium, (Figure 3). These results together with those measuring enzyme activities, indicate that under these conditions the glucocorticoid has no effect on vitamin B6 metabolism in these cells.

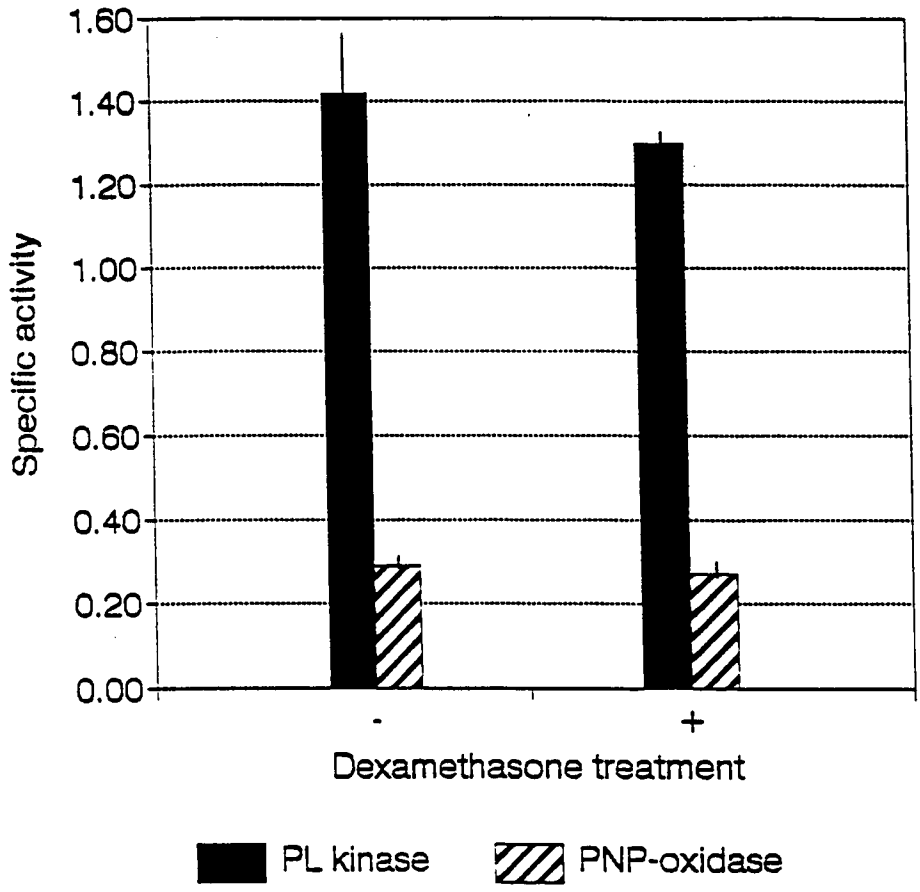

Figure 2. Pyridoxal kinase and PNP-oxidase activities in extracts of untreated cells and dex treated cells. Cells were cultured and treated with hormones as described in the methods. Cytosolic extracts of cells were prepared as described in Methods. Pyridoxal kinase and PNP-oxidase specific activities were measured as described. Dexamethasone treatment was for a period of 24 hours at a concentration of 10^{-6}M. Specific activity is expressed as nanomoles/min/mg. Each bar represents the average + standard deviation, n=9.

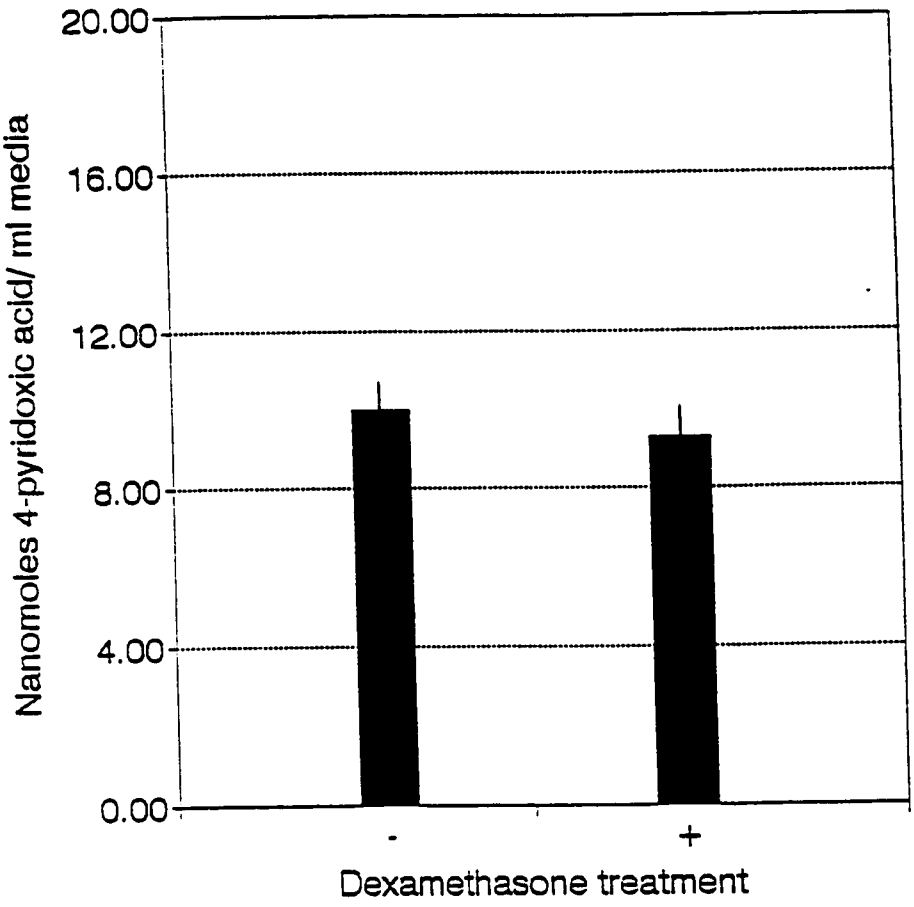

Figure 3. Extracellular 4-pyridoxic acid in the medium of untreated cells or dex treated cells. Cells were incubated and treated with hormone as described. 4-Pyridoxic acid was measured as described. Dexamethasone treatment was for a period of 24 hours at a concentration of 10^{-6}M. The accumulation of 4-pyridoxic acid into the media was for a period of 24 hours. Each bar represents the average + standard deviation, n=4.

Most of the studies describing PLP-mediated inhibition of steroid dependent gene expression have been performed by examining gene expression in cells which the vitamer concentrations are artificially manipulated. Artificial manipulation of the intracellular vitamer concentration was attempted by either growing cells in culture media devoid of any pyridoxal (PL-) or growing cells with pyridoxal but also incubated with 4-dPN a competitive inhibitor of pyridoxal kinase. Measurements made from extracts prepared from cells incubated under these two conditions are compared to those from cells that are grown with pyridoxal (PL). Two PLP dependent enzymes, tyrosine aminotransferase (TAT) and aspartate aminotransferase (AAT) are examined, as well as the induction of TAT activity by dexamethasone.

I examined pyridoxal kinase activity from extracts of cells grown in normal PL media, PL deficient media PL-, and in media supplemented with 4-dPN. A plot of pyridoxal kinase activity versus cell conditions is illustrated in Figure 4. In normal PL+ media, there is a decrease in activity from 0 day to 2 day ($p < .02$) and also a decrease from 0 day to 8 day ($p < .05$). In PL- deficient media, there is a decrease in activity from 0 day to 2 day ($p < .02$), 0 day to 4 day ($p < .005$), and 0 day to 6 day ($p < .005$). In 4-dPN media, there is a decrease from 0 day to 2 day and 0 day to 4 day ($p < .005$), and an increase from 2 day and 4 day to 6 day ($p < .005$). There is a decrease comparing 2 day PL+ to 2 day PL-, 4 day PL-, 6 day PL-, 2 day 4-dPN, and 4 day 4-dPN ($p < .001$).

TAT activity was assayed after artificially manipulating the vitamer concentration. The specific activity of TAT does not change significantly in extracts of cells maintained

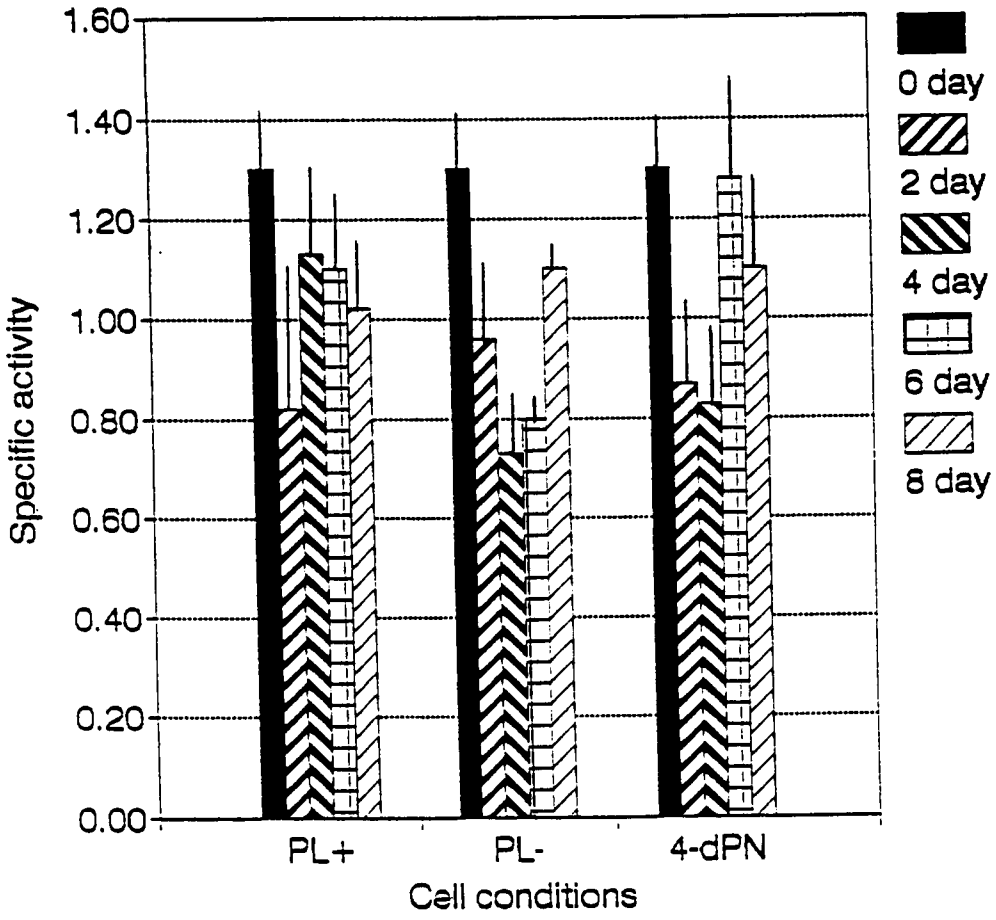

Figure 4. Basal pyridoxal kinase activity in extracts prepared from cells grown in normal, PL deficient, or 4-dPN supplemented media for up to 8 days. Cells were incubated and treated as described in the methods. Pyridoxal kinase activity was measured from extracts of nongrowing cells maintained in the following conditions: PL+, PL-, and 5 mM 4-dPN for 0, 2, 4, 6, and 8 days. Specific activity is expressed as nanomoles/min/mg. Each bar represents the average + standard deviation, n=8.

on normal PL+ media Figure 5. The activity of TAT from extracts of cells maintained on PL- media increases from 0 day to 8 day ($p < .001$). The activity of TAT from extracts of cells maintained on 4-dPN increases from 0 day to 2 day ($p < .005$), decreases from 2 day to 4 day ($p < .01$), increases from 4 day to 6 day ($p < .005$), and decreases from 6 day to 8 day ($p < .005$). TAT activity increases comparing 0 day PL+ to 8 day PL-, 2 day 4-dPN, and 6 day 4-dPN ($p < .001$).

The method presently available to measure the intracellular concentration of vitamin B6 i.e. the phenylhydrazine method (23), is not very sensitive. To determine if these cell treatments, PL- and 4-dPN, are depleting the cells of intracellular PLP we assayed the total and holo forms of the enzymes from extracts of cells grown PL+, PL-, and 4-dPN. After measuring both forms of the enzyme, the ratio of holoenzyme activity to total enzyme activity was calculated. For example, if the holo/total enzyme ratio of cells grown in media PL- decreases compared to cells grown PL+ this would indicate that enzyme from cells grown in PL- media were being depleted of cofactor. This ratio would indirectly inform us if intracellular PLP levels were decreasing in the cell.

The holoenzyme to total enzyme ratio of TAT in extracts of cells grown under these various conditions are illustrated (Figure 6). In extracts of cells grown in normal PL+ media there is an increase in the holo/total ratio from 0 day, 2 day, and 4 day to 6 day and 8 days ($p < .001$). In cells grown PL- deficient media there is a decrease from 0 day to 4 day ($p < .01$), 2 day to 4 day ($p < .001$), 6 day to 8 day ($p < .001$), and 0 day to 8 day ($p < .001$) and an increase from 4 day to 6 day ($p < .001$). In the case of 4-dPN an increase from 0 day to 4 day ($p < .01$), 4 day to 6 day ($p < .005$) and a decrease from

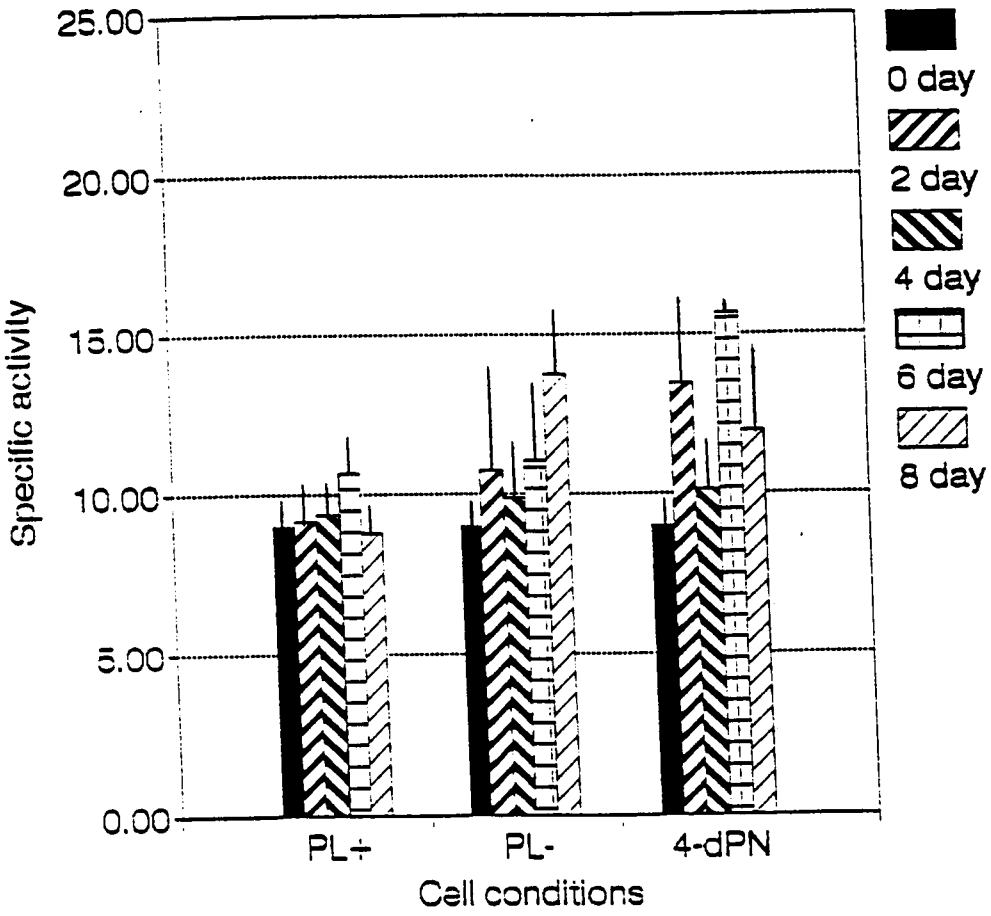

Figure 5. TAT basal enzyme activity in extracts prepared from cells grown in normal, PL deficient, or 4-dPN supplemented media for up to 8 days. Cells were incubated and treated as described in the methods. TAT activity was measured from extracts of nongrowing cells maintained in the following conditions: PL+, PL-, and 5 mM 4-dPN for 0, 2, 4, 6, and 8 days. Specific activity is expressed as nanomoles/min/mg. Each bar represents the average + standard deviation, n=8.

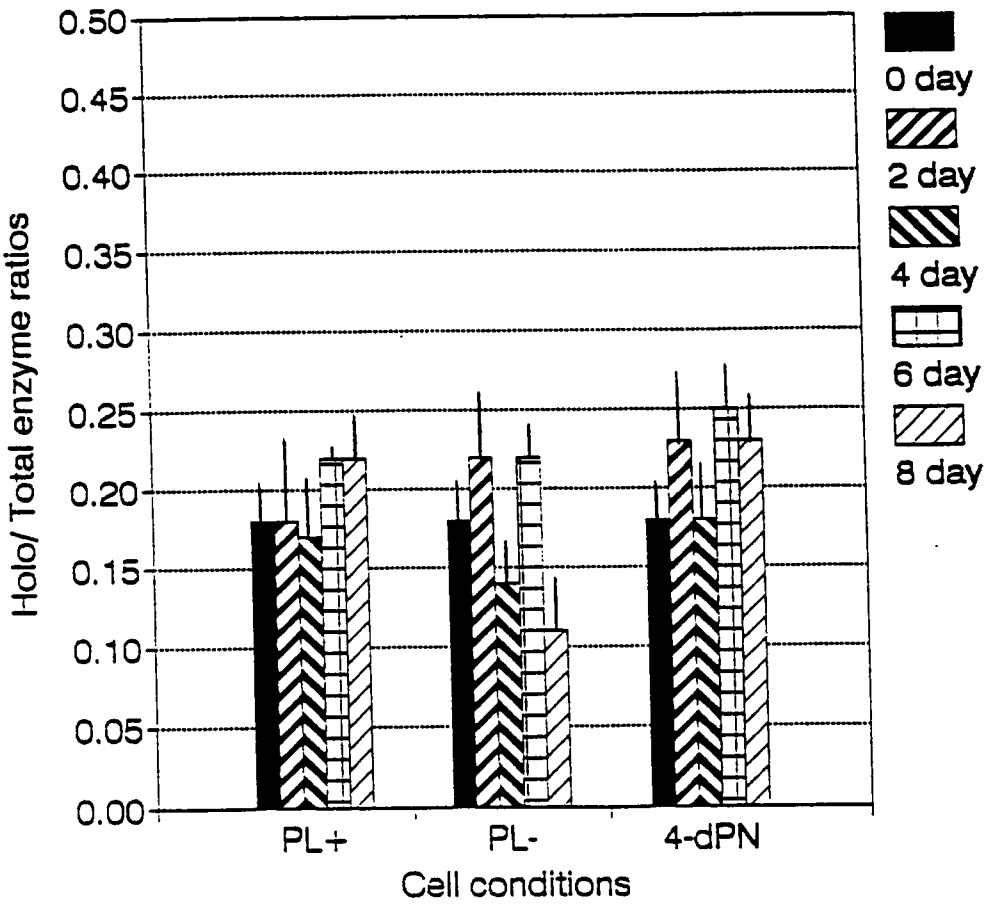

Figure 6. TAT holo/total enzyme activity ratios in extracts prepared from cells grown in normal, PL deficient, or 4-dPN supplemented media for up to 8 days. Cells were incubated and treated as described in the methods. TAT holo/total enzyme ratios were measured from extracts of nongrowing cells maintained in the following conditions: PL+, PL-, and 5 mM 4-dPN for 2, 4, 6, and 8 days. Each bar represents the average + standard deviation, n=8.

2 day to 4 day ($p<.01$). There is a decrease comparing 0 day PL+ to 8 day PL- ($p<.001$) and an increase from 0 day PL+ to 6 day PL-.

TAT also served as a positive control when we examined the effect of dex treatment on the activities of pyridoxal kinase and PNP-oxidase, and on the level of 4-pyridoxic acid. To be certain that dex was indeed having an effect on the cells we measured the activity of TAT from extracts isolated from control, untreated cells and extracts from dex-treated cells. The fold induction of TAT observed with the different incubation conditions is illustrated (Figure 7). There is an increase in the fold induction comparing the 2 day and 4 day to 6 day and 8 day ($p<.015$) of cells grown in normal media. In PL- deficient media, an increase from 2 day and 4 day to 6 day and 8 day ($p<.005$) is observed. In 4-dPN media no significant change was detected. There is a decrease in fold induction comparing the 2 day PL+ to the 2 day PL- ($p<.001$).

The activity of AAT has been reported to decrease in cells that have been depleted of pyridoxal (16). The AAT enzyme activity observed with the different incubation conditions is illustrated (Figure 8). In normal PL+ media there is an increase from 0 day to 4 day, 6 day, and 8 day ($p<.001$). In PL- deficient media there is an increase from 0 day to 6 day ($p<.001$). In 4-dPN media there is an increase from 0 day to 8 day ($p<.005$). There is a decrease in activity comparing 6 day PL+ and 8 day PL+ to 4 day PL-, 8 day PL-, 2 day 4-dPN, 4 day 4-dPN, and 6 day 4-dPN ($p<.001$).

The holoenzyme and total enzyme forms of AAT were assayed and there is no statistically significant change in the ratio of holo/total enzyme activity for AAT (Figure 9).

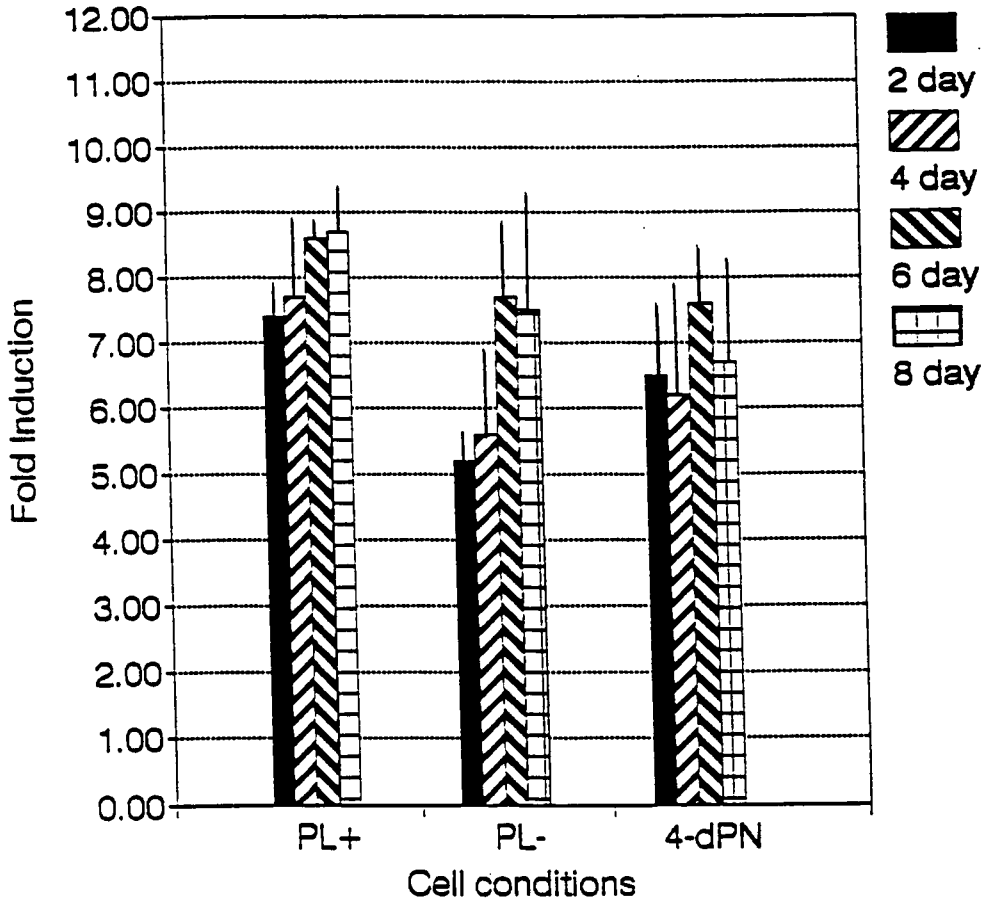

Figure 7. The induction of TAT by dex in extracts prepared from cells grown in normal, PL deficient, or 4-dPN supplemented media for up to 8 days. Cells were incubated and treated as described in the methods. TAT fold induction was measured from extracts of nongrowing cells maintained in the following conditions: PL+, PL-, and 5 mM 4-dPN for 2, 4, 6, and 8 days. Fold induction is defined as the specific activity of the dex treated sample divided by the specific activity of the untreated sample. Dexamethasone treatment was for a period of 24 hours at a concentration of 10^{-6}M. Each bar represents the average + standard deviation, n=4.

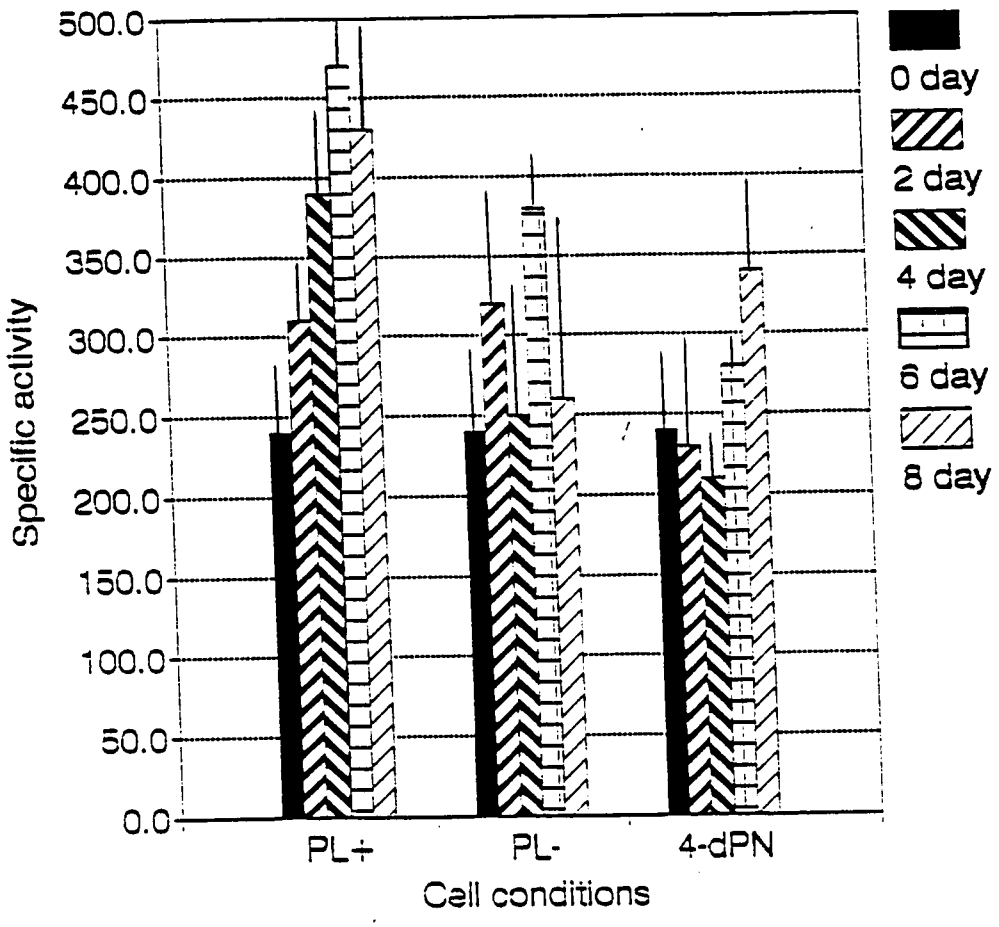

Figure 8. AAT basal enzyme activity in extracts prepared from cells grown in normal, PL deficient, or 4-dPN supplemented media for up to 8 days. Cells were incubated and treated as described in the methods. AAT activity was measured from extracts of nongrowing cells maintained in the following conditions: PL+, PL-, and 5 mM 4-dPN for 2, 4, 6, and 8 days. Specific activity is expressed as nanomoles/min/mg. Each bar represents the average + standard deviation, n=8.

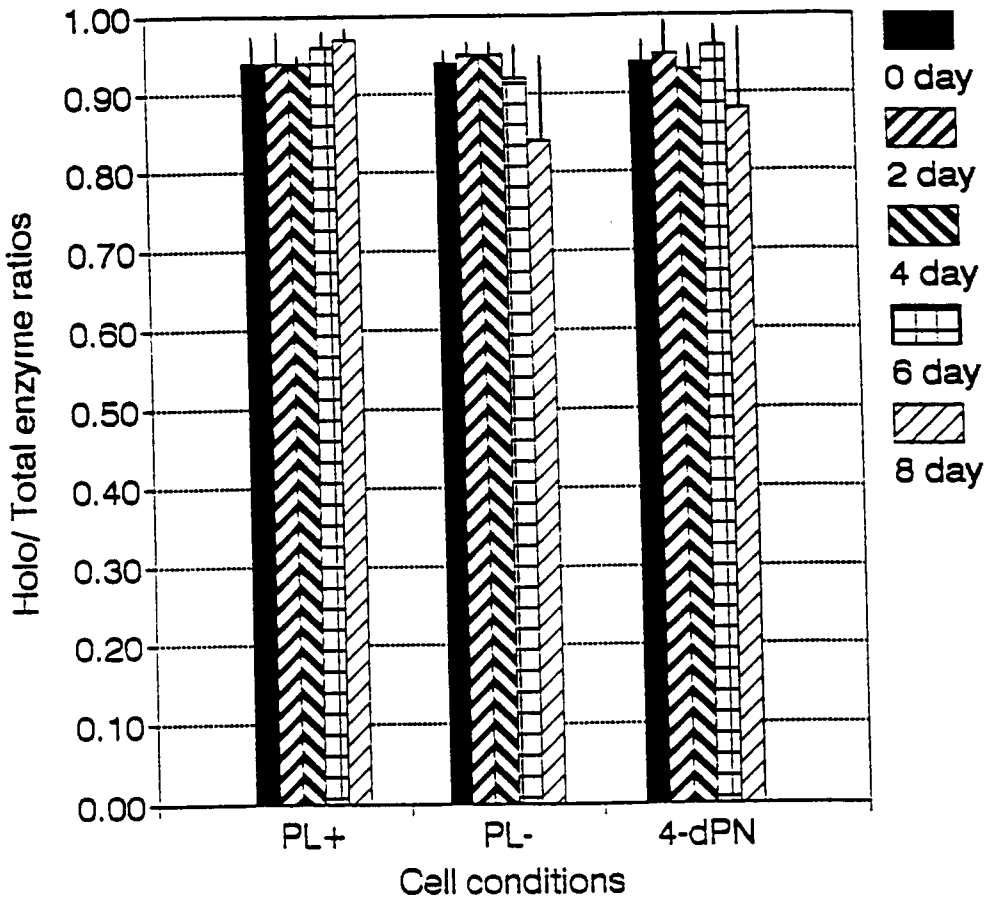

Figure 9. The AAT holo/ total enzyme activity ratios in extracts prepared from cells grown in normal, PL deficient, or 4-dPN supplemented media for up to 8 days. Cells were incubated and treated as described in the methods. AAT holo/ total enzyme ratios were measured from extracts of nongrowing cells maintained in the following conditions: PL+, PL-, and 5 mM 4-dPN for 2, 4, 6, and 8 days. Specific activity is expressed as nanomoles/min/mg. Each bar represents the average + standard deviation, n=8.

Enzyme Studies from Rapidly-growing Cells

These initial results differed from those reported in the literature (previously described in the introduction). I decided to attempt to explain this. The experiments in the literature were performed with cells that were constantly growing (7). My initial set of experiments were performed with non-growing quiescent cells. I considered this the most likely explanation for this discrepancy. In order to verify this hypothesis we repeated the above experiments using growing cells.

I first determined whether basal pyridoxal kinase activity is altered with the various manipulations. Pyridoxal kinase activity does not change significantly in extracts from cells grown in either PL+, PL- or 4-dPN media (Figure 10). The enzyme activity of pyridoxal kinase and the secretion of 4-pyridoxic acid were then measured in extracts from control cells and dex treated cells using growing cells at the 24 hour time point. Neither the activity of pyridoxal kinase (Figure 11) nor the secretion of 4-pyridoxic acid (Figure 12) changes significantly in response to dexamethasone treatment.

TAT basal activity was measured in extracts of growing cells. Figure 13 illustrates the basal TAT activity from cells grown in the various conditions. In normal PL+ media there is an increase from 0 day to 4 day, 6 day, and 8 day ($p < .001$). In deficent PL- media there is a increase from 0 day to 4 day, 6 day, and 8 day ($p < .001$). In 4-dPN media there is an increase from 0 day to 4 day, 6 day, and 8 day ($p < .001$). There is an increase in basal activity comparing the 8 day PL+ to the 8 day PL- ($p < .001$) and a decrease comparing the 8 day PL- to the 8 day 4-dPN ($p < .001$).

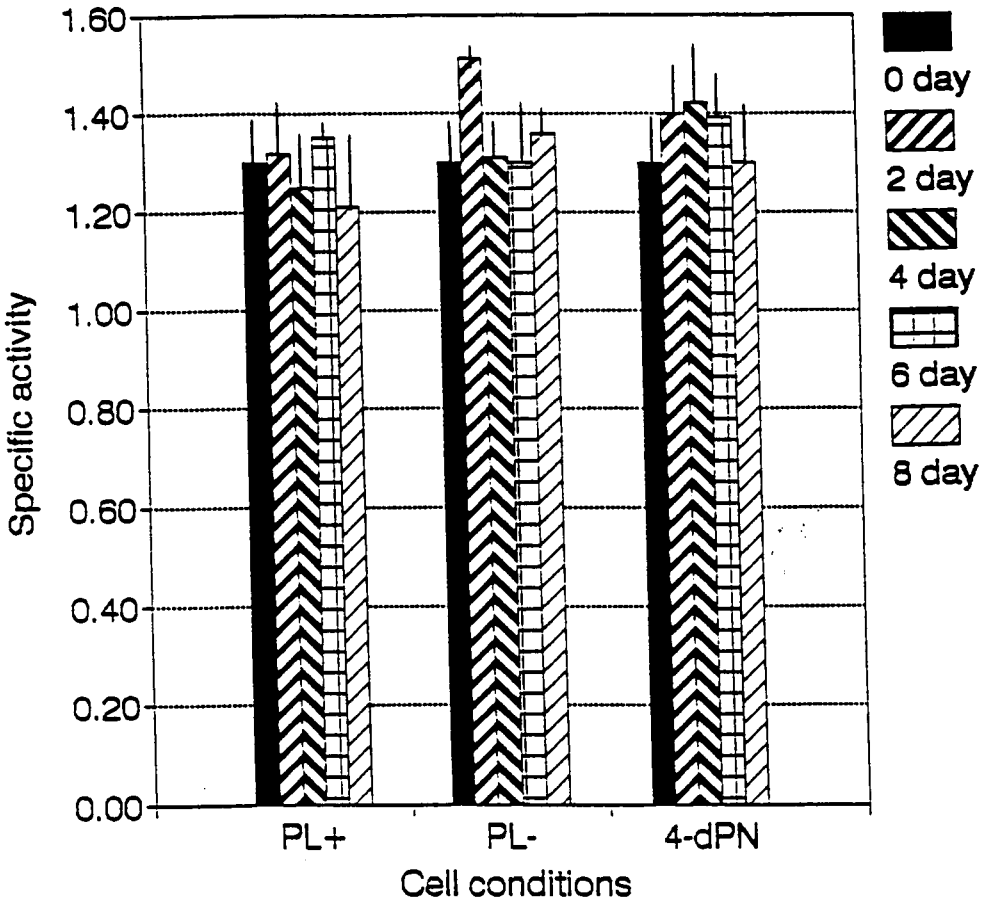

Figure 10. Basal pyridoxal kinase activity in extracts prepared from cells grown in normal, PL deficient, or 4-dPN supplemented media for up to 8 days. Cells were incubated and treated as described in the methods. Pyridoxal kinase activity was measured from extracts of rapidly growing cells maintained in the following conditions: PL+, PL-, and 5 mM 4-dPN for 2, 4, 6, and 8 days. Specific activity is expressed as nanomoles/min/mg. Each bar represents the average + standard deviation, n=8.

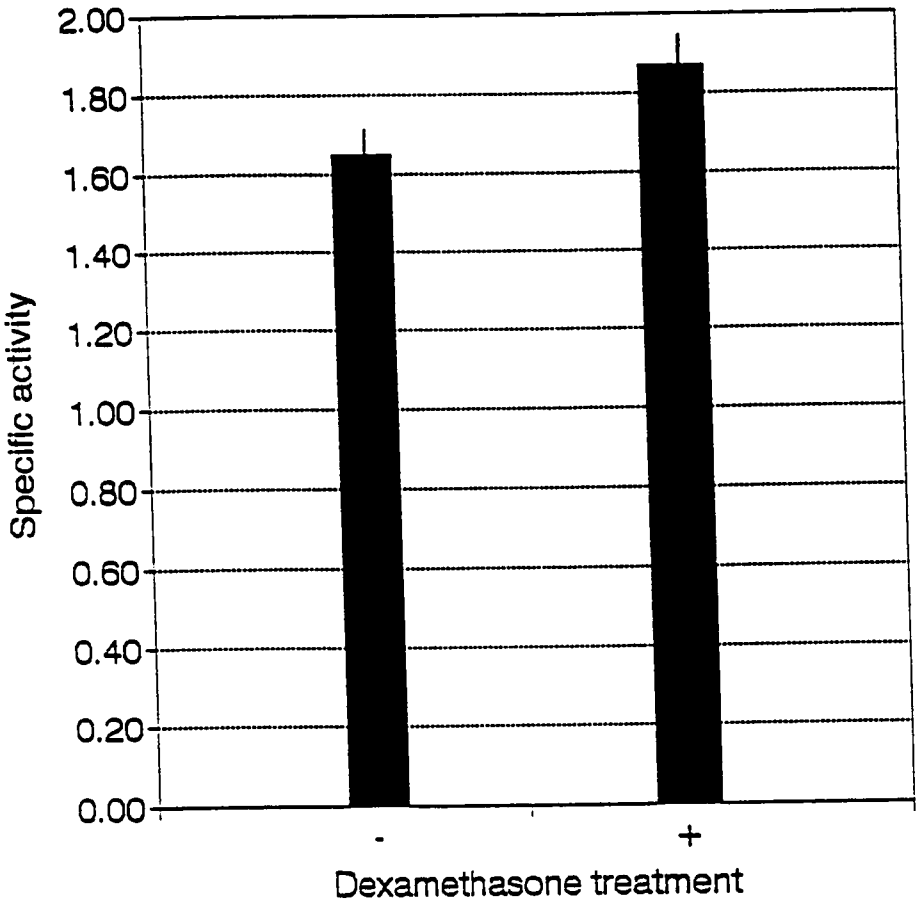

Figure 11. Pyridoxal kinase activity in extracts of untreated cells (-) and dex treated cells (+). Cells were incubated and treated and treated as described in the methods. Pyridoxal kinase specific activty was measured as described. Dexamethasone treatment was for a period of 24 hours at a concentration of 10^{-6}M. Specific activity is expressed as nanomoles/min/mg. Each bar represents the average + standard deviation, n=9.

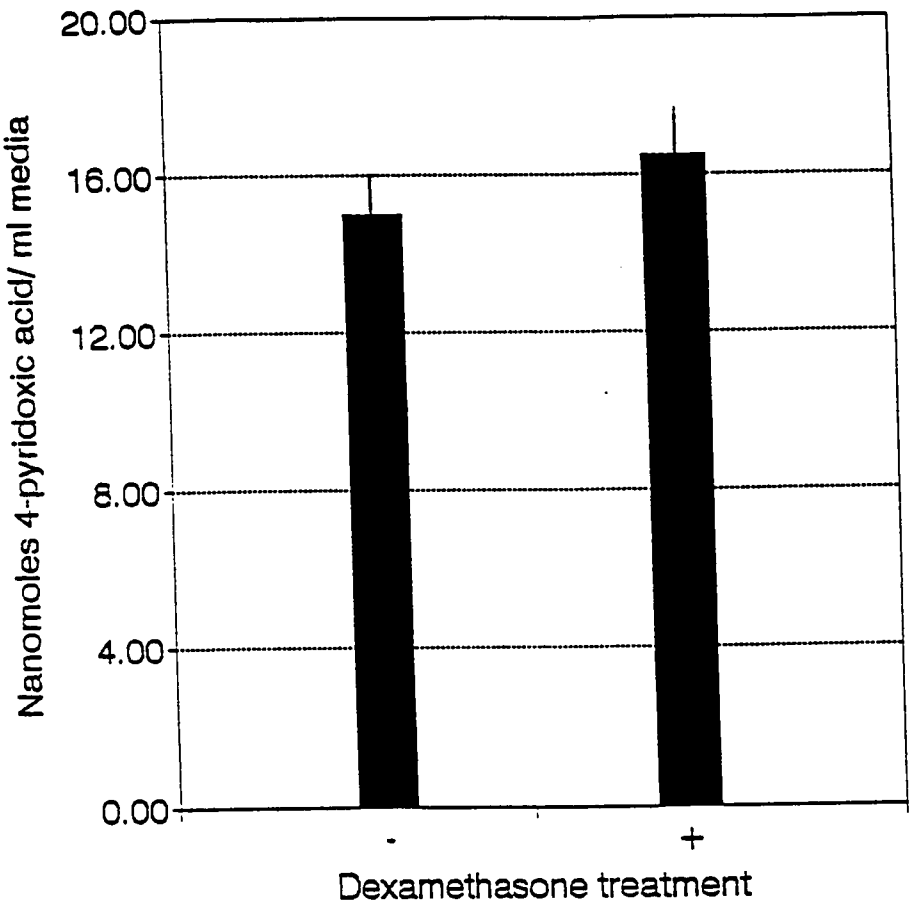

Figure 12. Extracellular 4-pyridoxic acid in the media of untreated cells (-) or dex treated cells (+). Cells were incubated and treated as described in the methods. 4-pyridoxic acid was measured from media of rapidly growing untreated cells (-) and in media from cells incubated with dexamethasone (+). Dexamethasone treatment was for a period of 24 hours at a concentration of 10^{-6}M. The accumulation of 4-pyridoxic into the media was for a period of 24 hours. Each bar represents the average + standard deviation, n=4.

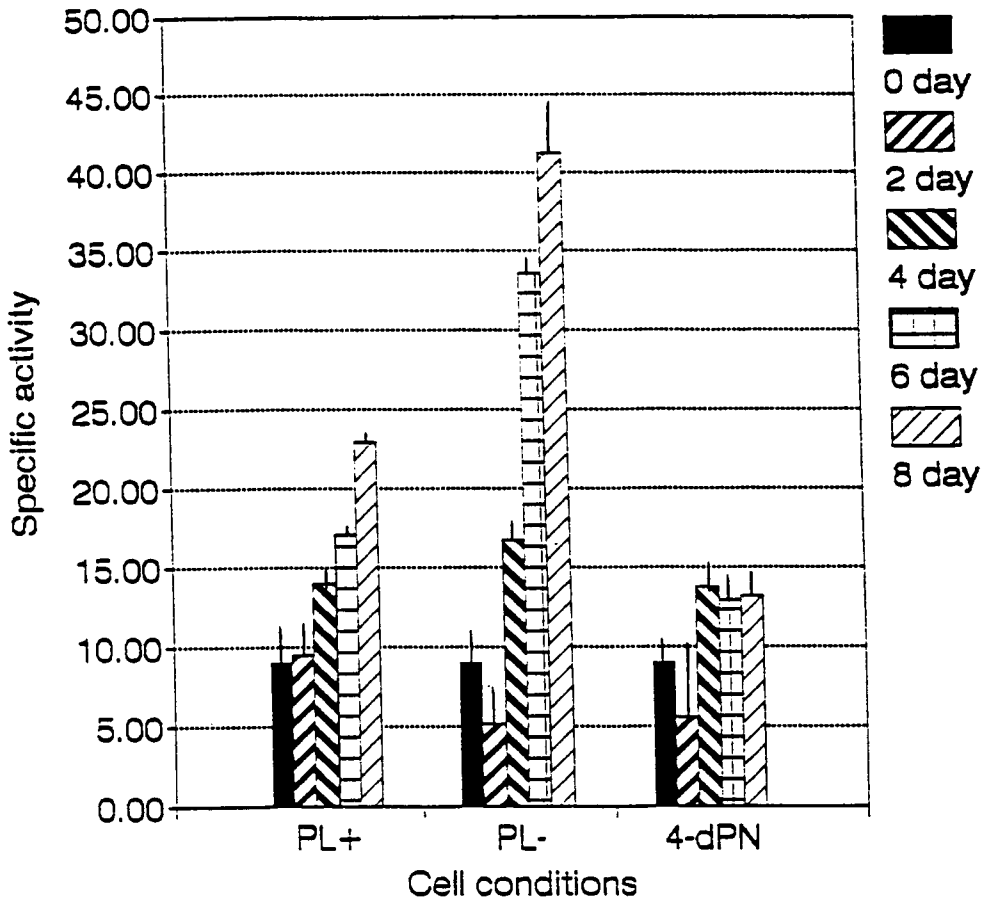

Figure 13. TAT basal enzyme activity in extracts prepared from cells grown in normal, PL deficient, or 4-dPN supplemented media for up to 8 days. Cells were incubated and treated as described in the methods. TAT activity was measured from extracts of rapidly growing cells maintained in the following conditions: PL+, PL-, and 5 mM 4-dPN for 2, 4, 6, and 8 days. Specific activity is expressed as nanomoles/min/mg. Each bar represents the average + standard deviation, n=8.

These results indicate that the basal levels of TAT are fluctuating in growing cells and are different from the results previously seen in the non-growing cells.

TAT holoenzyme to total enzyme ratios are illustrated in Figure 14. In normal PL+ media there is a decrease in the holo/total ratio from 0 day to 4 day, 6 day, and 8 day ($p < .001$). In deficient PL- media there is an increase from 0 day to 2 day ($p < .001$), a decrease from 0 day to 6 day ($p < .005$) and 0 day to 8 day ($p < .001$). In the case of 4-dPN media there is an increase from 0 day to 2 day and 6 day ($p < .001$). There is an increase comparing 8 day PL+ to 2 day 4-dPN, 4 day 4-dPN, 6 day 4-dPN, and 8 day 4-dPN ($p < .001$). Even though the ratio is decreasing in PL+ media there is a significant decrease comparing 8 day PL+ to 8 day PL-. These results suggest indirectly that the intracellular PLP concentration is decreasing in cells grown in PL deficient media.

The induction of TAT by dex was measured in extracts of growing cells, Figure 15. In normal PL+ media there is a decrease from 2 day to 4 day ($p < .02$). In deficient media PL- there is a decrease from 2 day to 4 day, 6 day, and 8 day. ($p < .001$). In 4-dPN treated media there is a decrease from 2 day to 4 day and 6 day ($p < .001$) and an increase from 4 day and 6 day to 8 day ($p < .001$). There is a decrease in the fold induction comparing 2 day PL+ to 4 day PL-, 6 day PL-, 8 day PL-, 4 day 4-dPN, and 6 day 4-dPN ($p < .001$). There is an increase in fold induction comparing 2 day PL+ to 2 day PL-, 2 day 4-dPN, and 8 day 4-dPN.

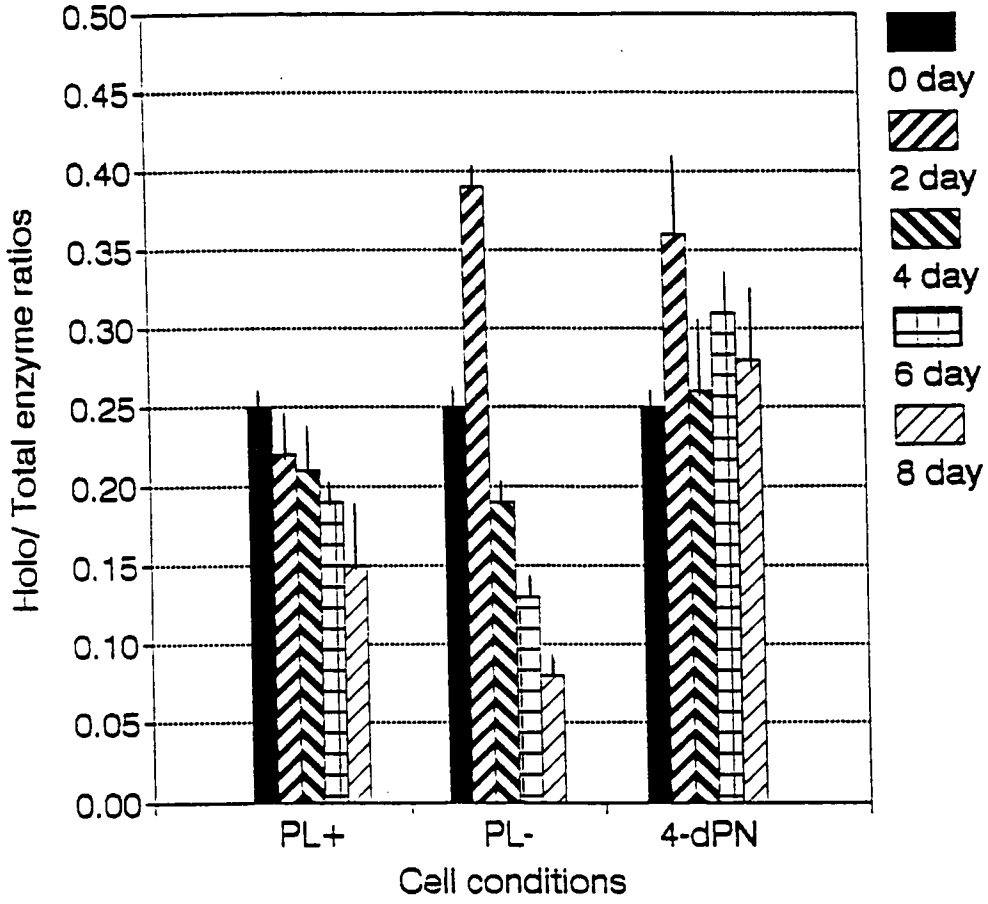

Figure 14. The TAT holo/ total enzyme activity ratios in extracts prepared from cells grown in normal, PL deficient, or 4-dPN supplemented media for up to 8 days. The TAT holo/ total enzyme activity ratios were measured from extracts of rapidly growing cells maintained in the following conditions: PL+, PL-, and 5 mM 4-dPN for 2, 4, 6, and 8 days. Each bar represents the average + standard deviation, n=8.

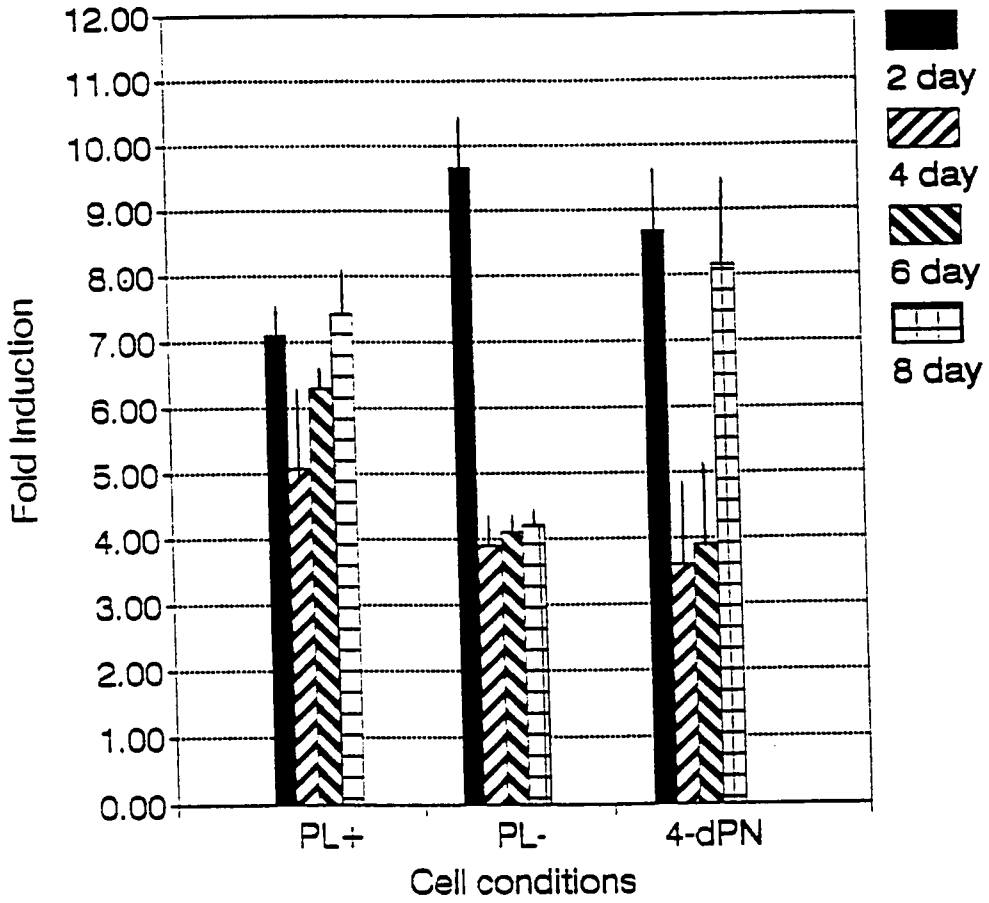

Figure 15. The induction of TAT by dex in extracts prepared from cells grown in normal, PL deficient, or 4-dPN supplemented media for up to 8 days. TAT fold induction was measured from extracts of cells maintained in the following conditions: PL+, PL-, and 5 mM 4-dPN for 2, 4, 6, and 8 days. Fold induction is defined as the specific activity of the dex treated sample divided by the specific activity of the untreated sample. Dexamethasone treatment was for a period of 24 hours at a concentration of 10^{-6}M. Each bar represents the average + standard deviation, n=4.

AAT specific activity was also assayed in extracts from growing cells (Figure 16). Basal AAT levels increase in normal PL+ media from 0 day and 2 day to 4 day and 6 day ($p < .001$). In deficient PL- media there is a decrease from 0 day and 2 day to 4 day, 6 day, and 8 day ($p < .001$). There is a decrease comparing all the PL+ days to 4 day, 6 day, and 8 day PL- ($p < .001$). We did not observe this decrease in AAT levels using nongrowing cells.This observation has been reported before (16). In 4-dPN treated media there is no significant change in the activity.

AAT holoenzyme to total enzyme ratios are illustrated in Figure 17. In normal PL+ media the holo/total enzyme ratios do not change significantly. In deficient PL- media, there is a decrease from 0 day, 2 day, and 4 day to 6 day and 8 day ($p < .001$). In 4-dPN treated media, there is no significant change in the ratios. There is a decrease comparing all the PL+ days to the 6 day and 8 day PL- ($p < .001$). This observation is another indication that incubation of growing cells in PL- media depletes cofactor from the cell. The observation that the holo/ total activity ratio is identical in control and 4-dPN treated cell extracts suggests that we do not observe depletion of intracellular PLP when growing the cells in 5 mM 4dxy.

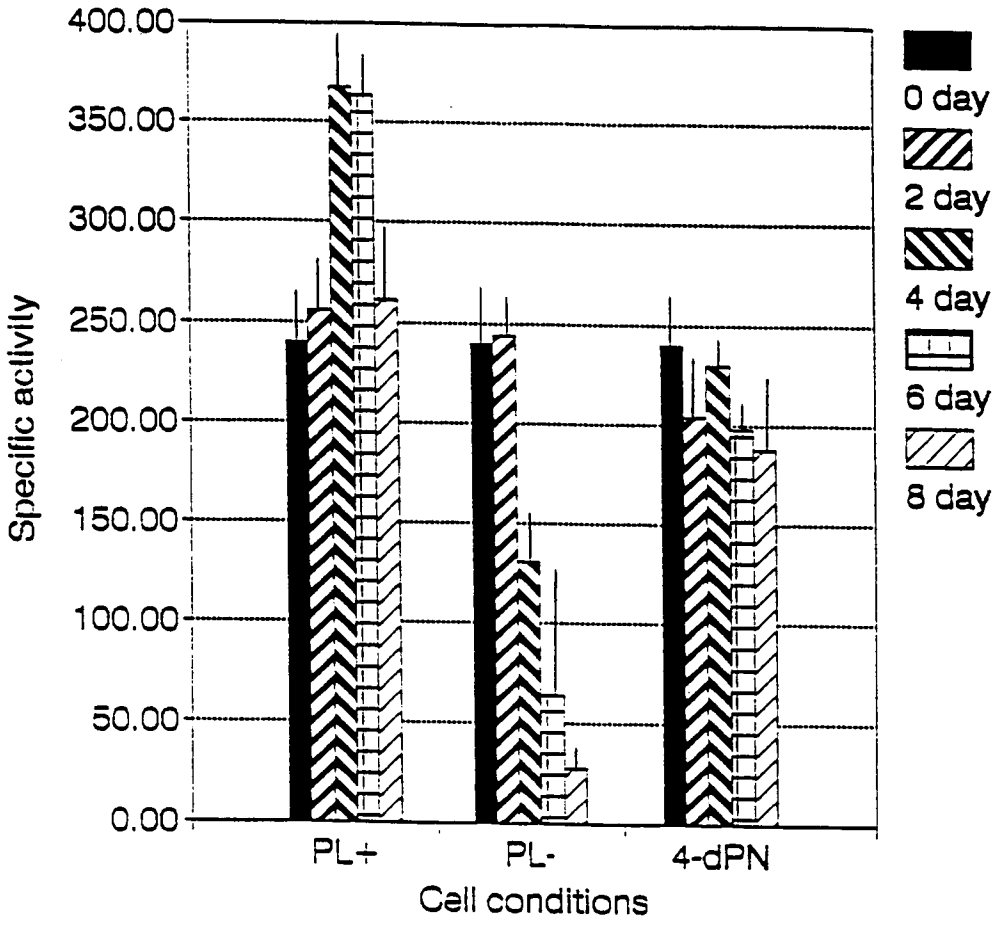

Figure 16. AAT basal enzyme activity in extracts prepared from cells grown in normal, PL deficient, or 4-dPN supplemented media for up to 8 days. AAT activity was measured from extracts of rapidly growing cells maintained in the following conditions: PL+, PL-, and 5 mM 4-dPN for 2, 4, 6, and 8 days. Specific activity is expressed as nanomoles/min/mg. Each bar represents the average + standard deviation, n=8.

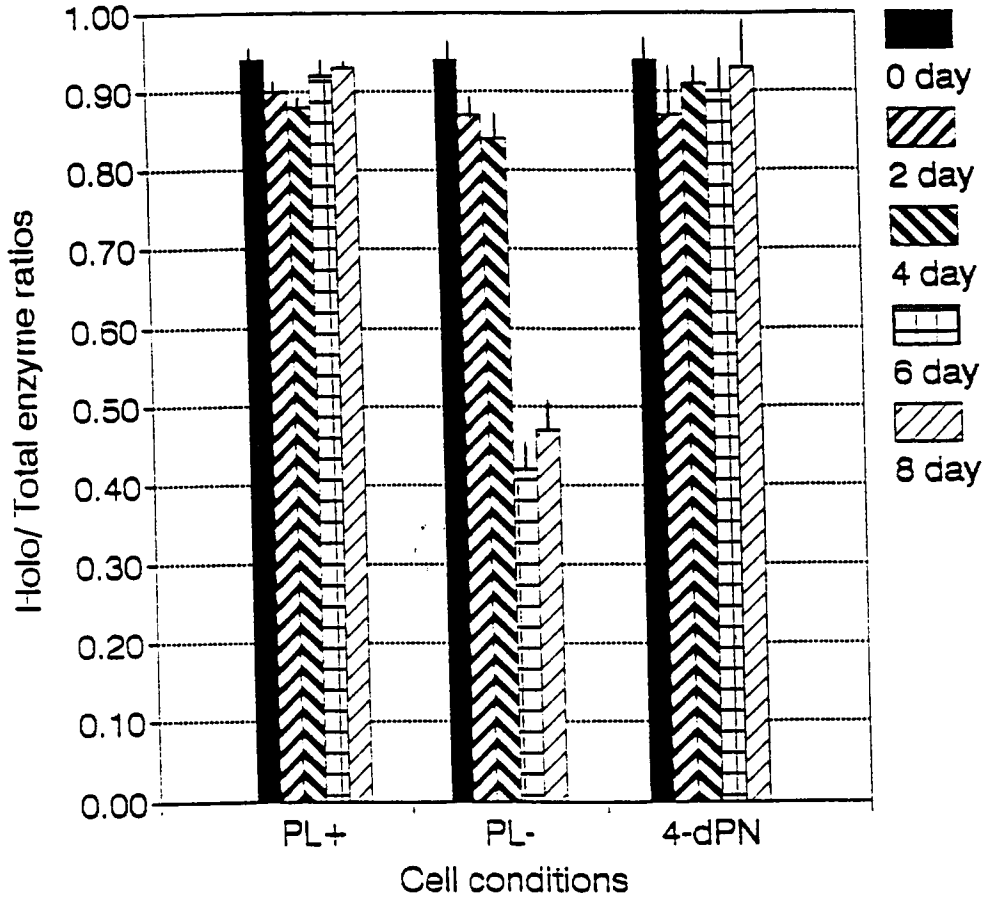

Figure 17. AAT holo/ total enzyme activity ratios from cells grown in normal, PL deficient, or 4-dPN supplemented media for up to 8 days. Cells were incubated and treated as described in the methods. AAT holo/ total ratios were measured from extracts of rapidly growing cells maintained in the following conditions: PL+, PL-, and 5 mM 4-dPN for 2, 4, 6, and 8 days. A value of $p < .05$ is indicated by an asterik. Each bar represents the average + standard deviation, $n = 8$.

DISCUSSION

The activities of pyridoxal kinase and PNP-oxidase as well as the release of 4-pyridoxic acid into the medium are no different in cells treated for 24 hours with dexamethasone than in untreated cultures. Pyridoxal kinase and PNP-oxidase are the two most important enzymes in the biosynthesis of PLP and 4-pyridoxic acid is the dead end catabolite of vitamin B6 metabolism. Since these are the rate limiting enzymes in PLP synthesis and the final breakdown product in PLP degradation, we can conclude that glucocorticoids do not effect vitamin B6 metabolism. These results lead us to conclude that the suggested functional relationship between steroid induction of gene expression of PLP dependent enzymes and PLP levels does not exist.

In the results statistical analysis was performed and certain numbers are statistically significantly different from others. In some instances, the biological importance of these statistical differences is not known. I have pointed out these differences in the results, however one cannot offer a plausible explanation for every one.

Basal pyridoxal kinase activity does not consistently change in non-growing cells maintained on normal PL+ media. There is a decline in activity at 2 days for both deficient PL- media and 4-dPN media. In the deficient PL- media the rebound of activity requires 6 days and in 4-dPN media the rebound in activity takes 4 days. There is no obvious explanation for this decline and rebound.

Basal TAT activity does not consistently change in non-growing cells maintained on normal PL+ media. In the deficient PL- media TAT activity gradually increases over 8 days. In 4-dPN media there is no consistent change.

TAT holo/total ratios do not change consistently in non-growing cells maintained on normal PL+ media, deficient PL- media, or 4-dPN media.

TAT fold induction by dex does not consistently change in non-growing cells maintained on normal PL+ media or 4-dPN media. The fold induction in deficient PL- media initially drops and then returns back to normal.

Basal AAT activity in non-growing cells maintained on normal PL+ media increases over the course of the experiment. In deficient PL- media or 4-dPN media there is no consistent change in AAT activity.

AAT holo/total ratios do not consistently change in non-growing cells maintained in normal PL+ media, deficient PL- media or 4-dPN media.

Basal pyridoxal kinase activity does not significantly change in growing cells grown in normal PL+ media, deficient PL- media or 4-dPN media.

Basal TAT activity steadily increases in growing cells grown in normal PL+ media. In deficient PL- media there is a drop in the 2 day activity and then a dramatic increase. In 4-dPN media there is no consistent change.

TAT holo/total ratios slowly decline in growing cells grown in normal PL+ media. In deficient PL- media the 2 day increases then a decline in the 4 day to 8 day. In 4-dPN media the 2 day, 4 day, and 6 day are similiar to PL- media, however, the 8 day is back up to the 2 day 4-dPN.

TAT fold induction by dex does not consistently change in growing cells grown on normal PL+ media. In deficient PL- media the 2 day increases and then the 4 day, 6 day, and 8 day are lower than the normal PL+ media.

Basal AAT activity increase at the 4 day and 6 day and then return to the 2 day level in growing cells grown on normal PL+ media. In deficient PL- there is a dramatic decline over the 8 day period. In 4-dPN media there is no consistent change.

AAT holo/total ratios in growing cells grown in normal PL+ media do not consistently change. In deficient PL- media there is a 50% decrease in the holo/total ratio comparing the 2 day and 4 day to 6 day and 8 day. In 4-dPN media there is no consistent change.

The non-growing cells and the growing cells yielded quite different results between TAT and AAT. The TAT holo/ total ratio decreases in PL- deficient media by 6 days in the growing cells, whereas the ratio in the non-growing cells decreases after 8 days. Furthermore the decrease in growing cells is greater than 60% whereas in the non-growing cells it only drops by about 30%. The TAT fold induction of the growing cells in PL- media decreases after 2 days, however, TAT fold induction increases in non-growing cells. AAT basal activity in PL- deficient media decreases in the growing cells, however, the AAT activity does not decrease in non-growing cells. The AAT holo/ total ratio in growing cells decreases, however the holo/ total ratio in growing cells does not decrease. All these results suggest that the growing cells are being depleted of cofactor faster than the non-growing cells. These results suggest a difference in the need for vitamin B6 between nongrowing and growing cells. Since the doubling time of the cells is 1 day and the cells are grown in PL- deficient media for 8 days the inside cellular contents of the vitamers are diluted 2^8 or 1:256 times.

The above described effects are only seen when the cells are grown in PL-deficient media and not when the cells are incubated with 4-dPN. In PL- media using growing cells the holo/total enzyme ratios for TAT and AAT decrease. Comparing PL+ media to 4-dPN media there are no significant changes in the holo/total ratios. The AAT activity levels decrease in PL- media using growing cells. Under the same conditions, except that treatment is with 4-dPN, there are no significant changes. These results suggest that deprivation of pyridoxal effects enzyme activities in a different manner than treating the cells with 4-dPN.

4-dPN is a substrate analog which closely resembles PLP. AAT apoenzyme is very unstable without PLP bound (described in the introduction). It is possible that 4-dPN binds to the newly synthesized enzyme and stabilizes the enzymes conformation. Since 4-dPN cannot be used as a cofactor the enzyme will be in a stable but inactive form. When excess PLP is added to assay for total enzyme activity the PLP could displace 4-dPN and allow the enzyme to be active. This is assuming that 4-dPN binds to the enzyme and PLP has a higher affinity for the enzyme than does 4-dPN.

Another difference between TAT and AAT are the holoenzyme to total enzyme ratios. 20% of TAT is in the holoenzyme form compared to 90% for AAT. This difference can be explained by differences in the affinity of these two enzymes for PLP. The Km of AAT for PLP has been reported to be 1 nM (5) while the Km of TAT for PLP has been reported to be 360 nM (6). Thus, AAT has a much higher apparent affinity for PLP than TAT. Since AAT has a greater affinity for PLP it would be

present mainly in the holoenzyme form providing that the intracellular concentration of PLP is high.

PART II.

PYRIDOXAL KINASE AND PNP-OXIDASE ASSAY

MATERIALS AND METHODS

Cell Culture

KRC-7 rat hepatoma cells were grown in monolayer culture in Dulbecco's modified Eagle's medium containing 7.5% v/v iron-supplemented calf serum (Hyclone), penicillin (50 units/ml), and streptomycin (0.1 mg/ml), at 37° C in a humidified atmosphere of 90% air, 10% CO_2.

Preparation of Cell Extracts

The media on confluent KRC-7 cells was aspirated. The cells were washed with an ice cold phosphate buffered saline (PBS) (10mM $NaKHPO_4$ at pH 7.4, 130mM NaCl, 5mM KCl). The cells were collected in PBS and transferred to a microfuge tube. Cells were lysed by adding NP-40 to a final concentration of 0.1% (v/v) and mixing. The solution was centrifuged for 15 min at 15,000 rpm and the supernatant was assayed for enzyme activity.

Assay Procedure

The enzyme containing solution was added to the substrate solution described in (28) to start the reaction. Briefly, this is an 0.1M potassium phosphate solution at pH 6.4 containing: 0.2mM pyridoxal, 1mM ATP, and 0.1mM zinc. This mixture was incubated for the indicated times, with shaking at 37° C. The sample was transferred to a cuvette to assay for the presence of PLP. Hydroxylamine was added to a final concentration of 1mM and the fluorescence was recorded immediately using an excitation

wavelength of 380nm and an emission wavelength of 450nm. Fluorescence measurements were carried out in a Perkin Elmer LS 50B fluorimeter.

Protein concentrations were determined by the method of Bradford (30).

RESULTS

The fluorescence emission spectrum of pyridoxal or pyridoxal-5-phosphate reacted with hydroxylamine is illustrated in Figure 18. PL and PLP alone do not fluoresce under these experimental conditions; i.e., excitation 380, emission 450. However, formation of the oximes of PL and PLP results in a dramatic increase in the fluorescence. The graph represents the oximes after allowing for complete oxime formation. Since both oximes have the same emission wavelenth maximium, 450nm, they cannot be distinguished using this property.

The rates of oxime formation for PL and PLP were studied to determine if this would allow us to distinguish between the two oximes. The rates of formation of both PL-oxime and PLP-oxime are shown in Figure 19. These results indicate that formation of the PLP-oxime is more rapid than formation of the PL-oxime. The PLP-oxime formation is very rapid and complete after 2.5 minutes compared to 120 minutes for the complete PL-oxime formation. Thus, the two oximes can be distinguished by studying the kinetics of their formation.

We determined the pseudo first order rate constants for PL-oxime and PLP-oxime formation. This was performed by recording the fluorescence due to oxime formation versus time using various PL or PLP concentrations. The slope of the initial rate of formation was plotted versus PL and PLP concentration (Figure 20). The ratio of PL:PLP pseudo first order rate constants is 1:48.

To demonstrate that PLP-oxime formation is concentration- dependent, hydroxylamine was reacted with varying concentrations of PLP. The data in Figure 21

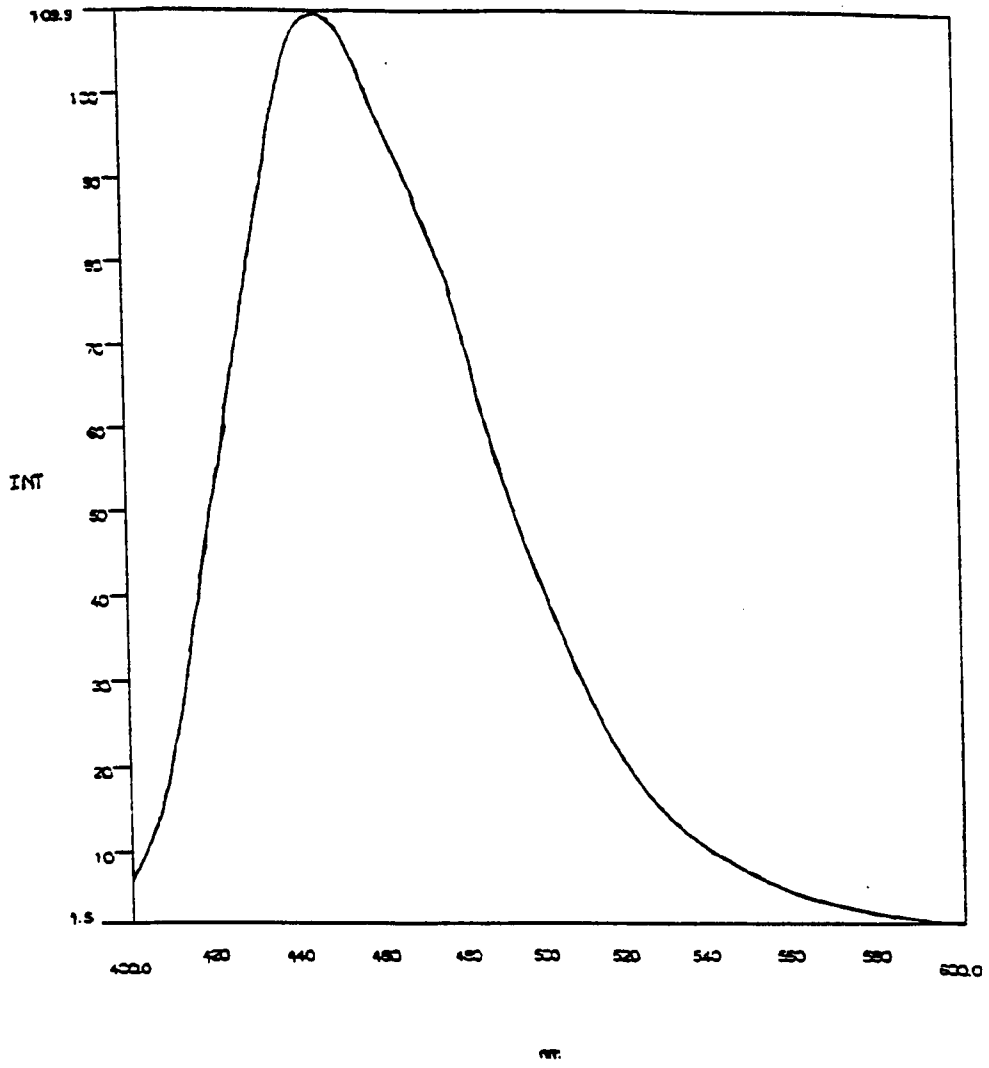

Figure 18. Fluorescence emission spectrum of PL-oxime or PLP-oxime. Hydroxylamine is added to a final concentration of 1mM to a solution of PL or PLP, each at 5 uM. The excitation wavelength is 380nm. The fluorescence emission is recorded at 450nm.

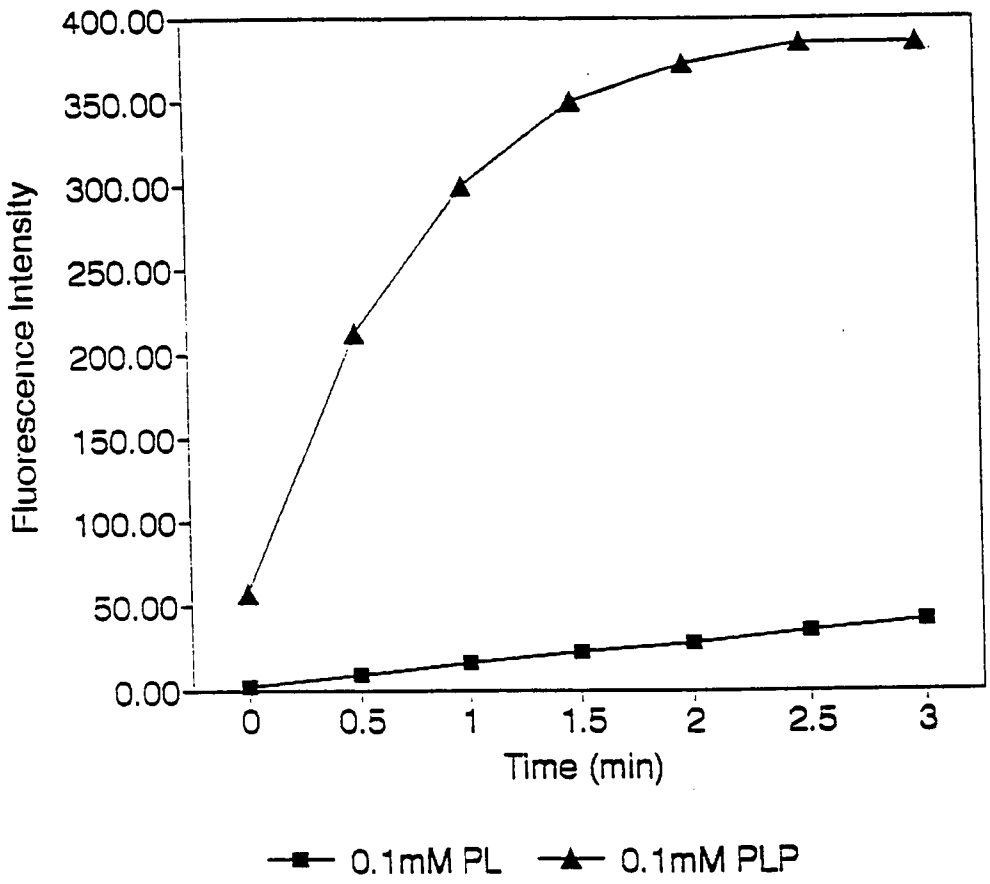

Figure 19. Rate of formation of the oxime derivatives following addition of hydroxylamine. Hydroxylamine is added to a final concentration of 1mM to a solution of PL or PLP, each at 0.1 mM. The excitation wavelength is 380nm. The fluorescence emission is recorded at 450nm.

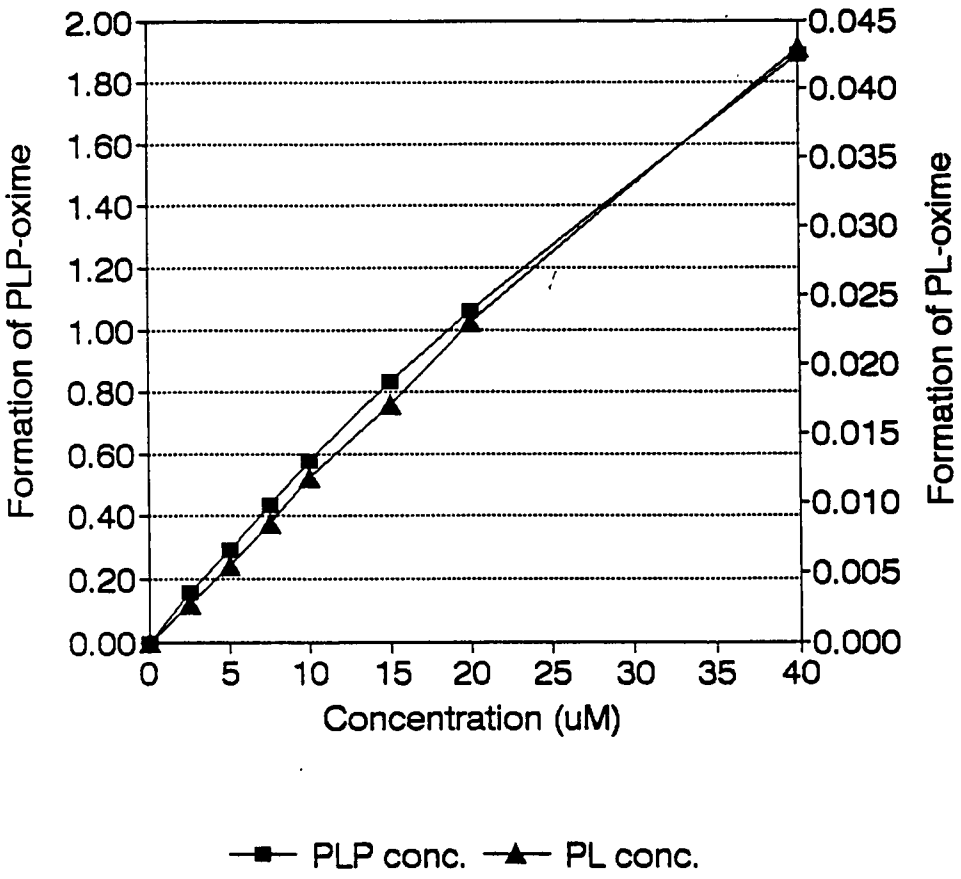

Figure 20. Initial velocity of formation of oximes of PL or PLP. Hydroxylamine was added to a final concentration of 1 mM to the indicated concentrations of PL and PLP. The fluorsecence was recorded versus time. The initial slope of the line was calculated and was replotted versus concentration of PLP (squares) and PL (triangles).

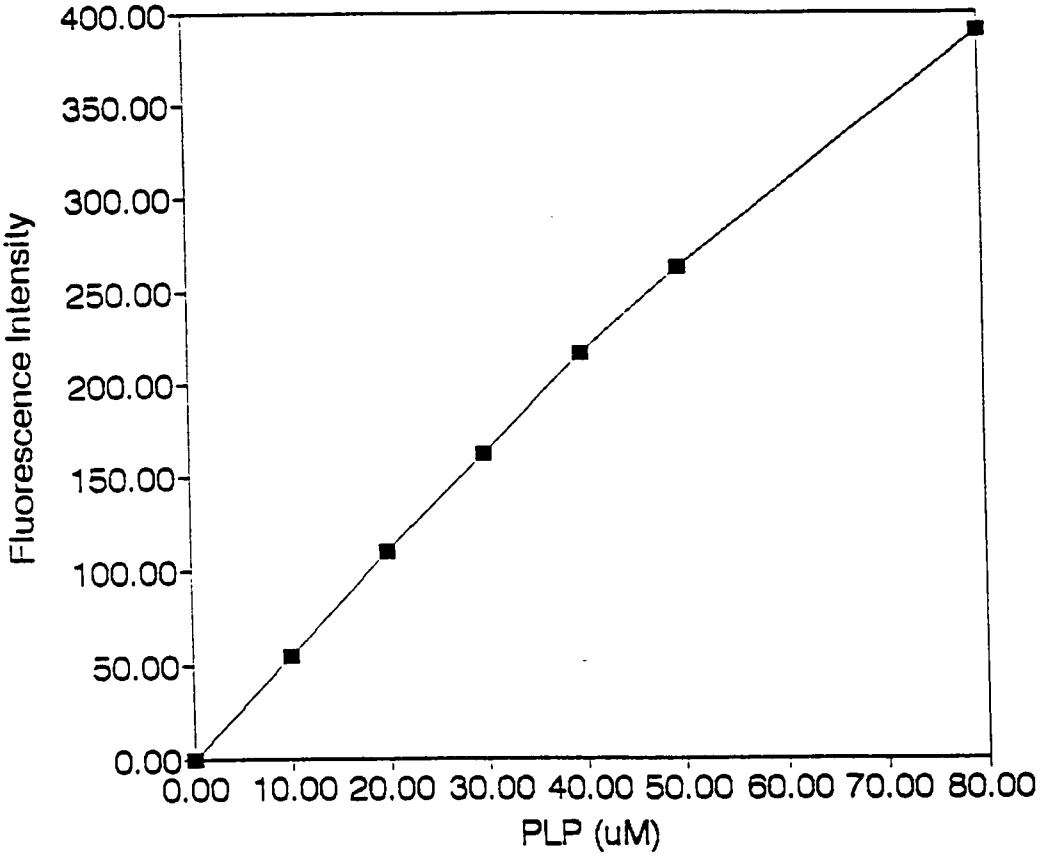

Figure 21. The fluorescence due to PLP-oxime is dependent on the initial concentrations of PLP. Hydroxylamine was added to a final concentration of 1 mM to increasing concentrations of PLP. The fluorescence intensity was recorded with time. The data represents the fluorescence due to the formation of the oximes at the indicated concentrations 2.5 minutes after the addition of hydroxylamine.

indicate a linear increase in fluorescence when varying concentrations of PLP are reacted with hydroxylamine. These results demonstrate linearity in the absence of PL.

To determine whether a similar linearity would be obtained when the reaction is performed in the presence of PL, various concentrations of PLP were added to an 0.2 mM PL solution. Hydroxylamine was added and the rate of increase in fluorescence was recorded. The results shown in Figure 22 demonstrate that after subtracting the fluorescence due to the PL oxime alone, there is a linear dependence between the fluorescence and the concentration of PLP in the reaction mixture.

To demonstrate that this assay could be used to measure pyridoxal kinase activity using a purified preparation of the enzyme. We tested two different amounts and 4 different incubation times of the purified enzyme. The results shown in Figure 23 demonstrate that the formation of the PLP-oxime is dependent upon the amount of enzyme used and the time of incubation.

The linearity with respect to time and protein concentration using crude enzyme in cell extracts was tested. Linearity with respect to time and protein concentration are observed by measuring pyridoxal kinase from cultured hepatoma cells Figure 24.

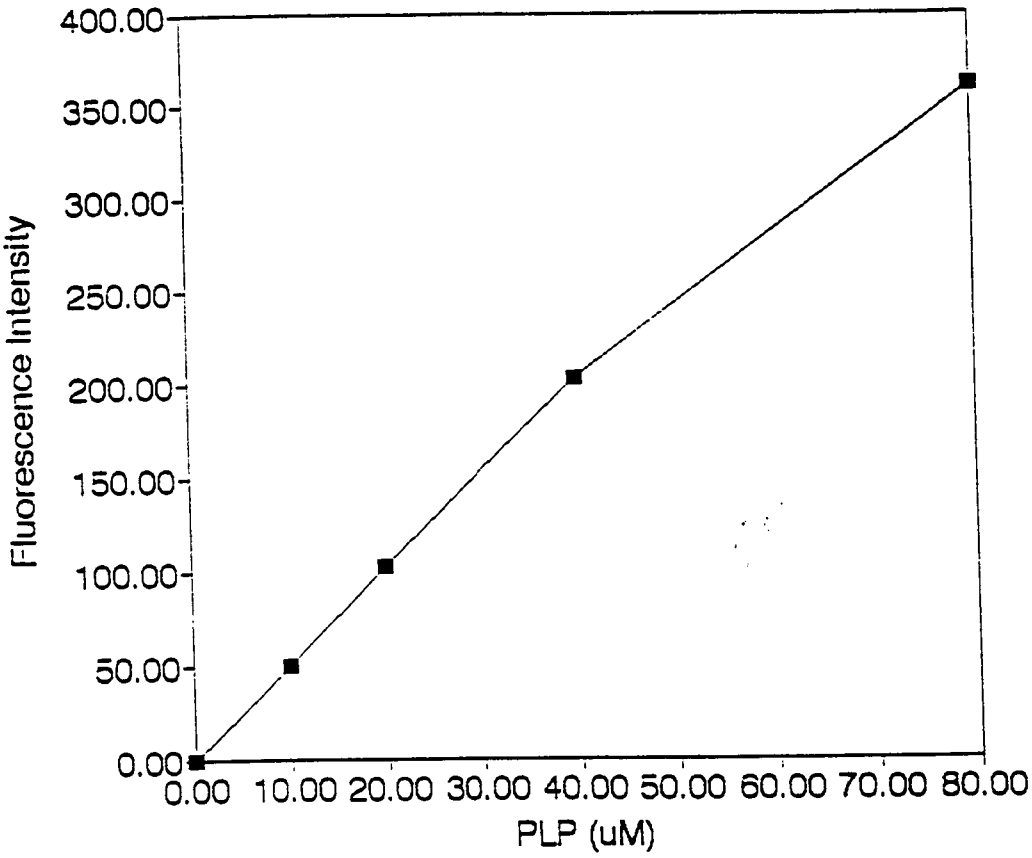

Figure 22. The fluorescence due to the PLP-oxime is concentration dependent in the presence of 0.2mM PL. Solutions were prepared containing the indicated concentrations of PLP, each in the prescence of 0.2mM PL. Hydroxylamine was added to a final concentration of 1mM and the recording of the fluorescence emission at 450nm begun immediately. Separately, a reaction with 0.2mM PL alone was recorded. The data presented represents the fluorescence intensity of the individual samples containing PLP and PL minus the fluorescence intensity of PL alone; I_{PL+PLP}-I_{PL}, measured at 2.5 minutes.

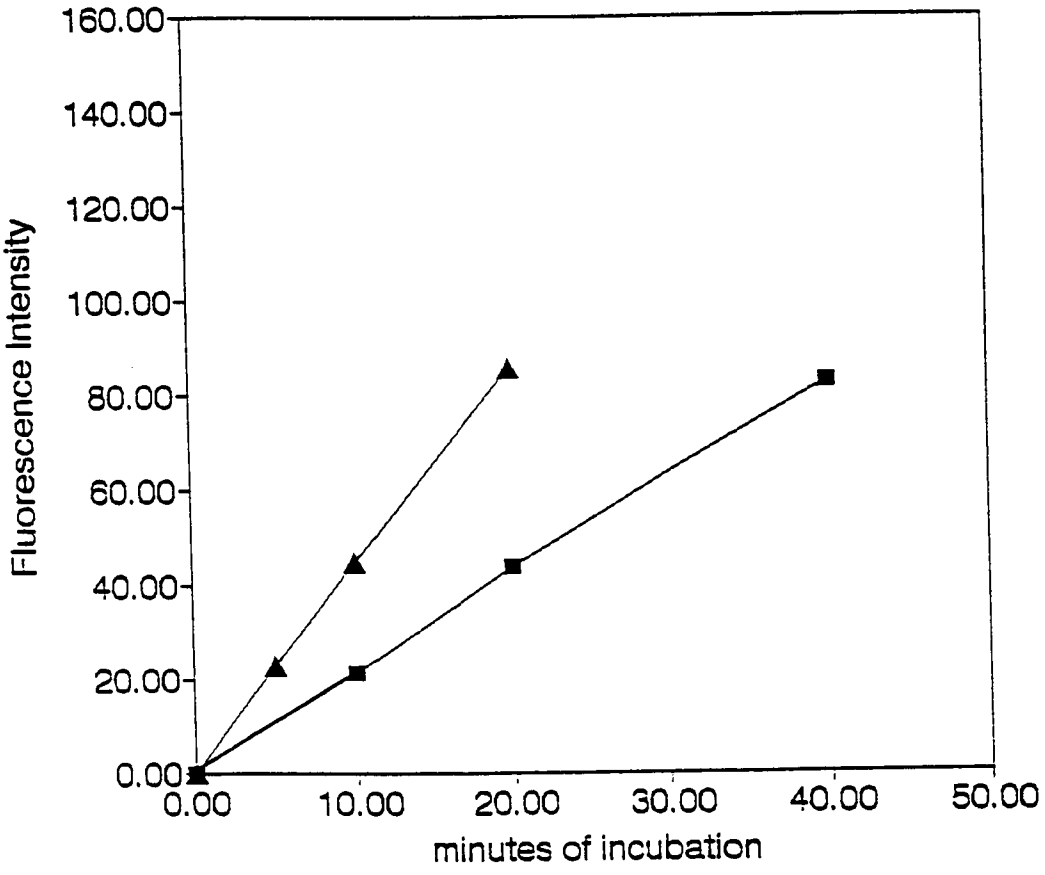

Figure 23. Dependence of PLP-oxime formation on the amount of enzyme and time of incubation with purified pyridoxal kinase. The reactions were performed as described in materials and methods. All lines represent the fluorescence intensity of PLP, I_{PL+PLP}-I_{PL} after 2.5 minutes. Pure pyridoxal kinase (2.5 ug) represented by square markers and (5.0 ug) represented by triangles. The concentration of PLP formed by the enzyme can be calculated by using the following equation:$[(I_{sample}-I_{0.2\ mM\ PL})/\ (I_{0.2\ mM\ PLP})]$ *.2 mM.

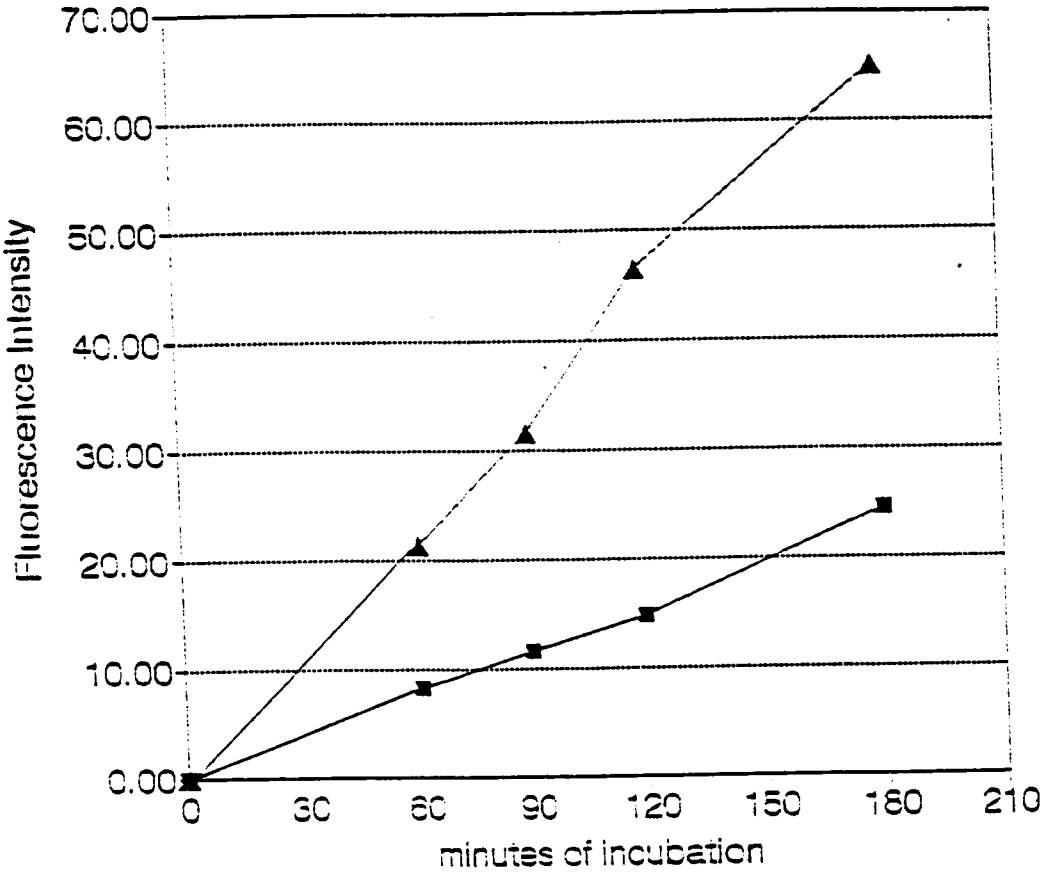

Figure 24. Dependence of PLP-oxime formation on the amount of enzyme and time of incubation with crude extracts prepared from cultured rat hepatoma cells. Crude cytosolic extracts from rat hepatoma cells were prepared as described in materials and methods. Assays were performed as described in the legend to Figure 5. Crude extracts (75 ug) represented by squares. Crude extracts (150 ug) represented by triangles.

DISCUSSION

Our initial attempt to assay pyridoxal kinase in cultured hepatoma cells proved unsuccessful using both the phenylhydrazine assay and the direct colorimetric assay. To avoid the use of radioisotopes we developed a new assay that is very sensitive in determining low levels of PLP within a background of PL and does not require extra separation procedures. The method is very easy to use and requires inexpensive materials.

LIST OF REFERENCES

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1) Buss, D., Hamm, M., Mehanso, H., and Henderson, L. (1980) The Journal of Nutrition 110(8):1655-1663.

2) Mehanso, H., Hamm, M.m and Henderson, L. (1979) The Journal of Nutrition 109(9):1542-1551.

3) Allgood, V., Powell-Oliver, F., and Cidlowski, J. (1990) Journal of Biological Chemistry 265(21):12424-12433.

4) Dolphin, D., Poulson, R., and Avramovic, O. (1986) Vitamin B6 Pyridoxal-5-Phosphate: Chemical, Biochemical, and Medical Aspects. p.449-476.

5) Churchich, J.E. (1972) Journal of Biological Chemistry 247:4192-4196.

6) Koontz, J., and Wicks, W. (1984) Journal of Biological Chemistry 259:929-934.

7) Lipson, M., Kraus, J., Solomon, L., and Rosenburg, L. (1980) Archives of Biochemistry and Biophysics 204(2):486-493.

8) Shibuya, M., Nagata, K., and Okada, M. (1982) J. Biochem. 92:1399-1402.

9) Churchich, J.E. (1972) Journal of Biological Chemistry 247(13):4192-4196.

10) Masugi, F., Sumi, Y., Shimizu, S., and Fukui, S. (1973) Journal of Nutrition and Science Vitaminology 19:229-236.

11) Fonda, M., Trauss, C., and Guempel, U. (1991) Archives of Biochemistry and Biophysics 288:79-86.

12) Lee, K.L., Darke, P.L., and Kenney, F.T. (1977) Journal of Biological Chemistry 252:4958-4961.

13) DiSorbo, D., and Litwack, G. (1981) Biochemical and Biophysical Research Communications 99(4):1203-1208.

14) Muller, M. and Renkawitz, R. (1991) Biochimica et Biophysica Acta1088:171-182.

15) Schmid, E., Wolfgang, S., Jantzen, M., Mayer, D. Jarstoff, B. and Schutz, G. (1987) Eur. J. Biochem. 165:499-506.

16) Barouki, J., Pave-Preux, M., Bousquet-Lemercier, B., Pol, S., Bouguet, J., and Hanoune, J. (1989) Eur. J. Biochem 186:79-85.

17) Katunuma, N., and Kobayashi, K. (1978) Morris Hepatomas Mechanism of Regulation Advances in Experimental Medicine and Biology Vol.92:665-674.

18) Lee, K.L., Kenney, F.T. (1970) Biochemical and Biophysical Research Communications 40(2):469-475.

19) Sawaki, S., and Hattori, H. (1970) Journal of Vitaminology 16:83-85.

20) Allgood, V., and Cidlowski, J. (1992) Journal of Biological Chemistry 267(6):3819-3824.

21) Maksymowych, A., Daniel, V., and Litwack, G. (1990) Annals of New York Academy of Sciences 585:438-451.

22) Meisler, N.T., and Thanassi, J.W. (1980) Analytical Biochemistry 90:525-533.

23) Wada, H. and Snell, E.E. (1961) Journal of Biological Chemistry 236:6489-6495.

24) Kwok, F. and Churchich, J.E. (1979) Journal of Biological Chemistry 254:6489-6495.

25) Beeler, T. and Churchich, J.E. (1976) Journal of Biological Chemistry 251:5267-5271.

26) Diamondstone, T. (1966) Analytical Biochemistry 16:395-401.

27) Karmen, A., Wroblewski, F., and La Due, J. (1956) Journal of Clinical Investigation 61:645-653.

28) Churchich, J., Scholz, G., and Kwok, F. (1989) Biochimica et Biophysica Acta 996:181-186.

29) Meisler, N.,and Thanassi J. (1980) Journal of Nutrition 110:1965-1975.

30) Bradford, M. (1976) Analytical Biochemistry 72:248-254.

31) Contractor, S., and Shane, B. (1968) Clinica Chimica Acta 21:71-77.

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