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5′-Deoxypyridoxal Interaction with Dexamethasone Receptor: A New Probe for Structure and Function of Steroid Receptors†

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ABSTRACT: 5′-Deoxypyridoxal, a vitamin B-6 analogue, increased the rate of dissociation of [^{3}H]dexamethasone from HeLa S_3 cytoplasmic glucocorticoid receptor complexes in vitro. This effect was achieved at millimolar concentrations of 5′-deoxypyridoxal, suggesting a low-affinity interaction of 5′-deoxypyridoxal with receptor. Loss of [^{3}H]dexamethasone-receptor binding in the presence of 5′-deoxypyridoxal was pH dependent, and a plot of K_{diss} vs. pH fit a simple sigmoidal titration curve with an inflection point at pH 7.8, suggesting that deprotonation of a single functional group on 5′-deoxypyridoxal increases K_{diss}. Loss of [^{3}H]dexamethasone binding in the presence or absence of unlabeled steroid also increased with pH, but no inflection point occurred over the range of pH tested. A titration of 5′-deoxypyridoxal indicated a pK of 7.94 for the pyridinium proton, suggesting deprotonation of the pyridinium nitrogen may account for the pH dependence of K_{diss} of dexamethasone from receptor. 5′-Deoxypyridoxal also caused a decrease in nuclear [^{3}H]-dexamethasone-receptor binding when incubated with whole HeLa S_3 cells at 37 °C. Furthermore, 5′-deoxypyridoxal was effective in reducing nuclear binding of dexamethasone when added either simultaneously with [^{3}H]dexamethasone or after achievement of equilibrium of steroid with receptor. The reduction in nuclear [^{3}H]dexamethasone binding is highly specific for 5′-deoxypyridoxal. Several analogues of this compound, including 5′-deoxypyridoxamine, were ineffective. In addition, this effect was reversible following removal of extracellular 5′-deoxypyridoxal. Under these conditions, 5′-deoxypyridoxal was competitive with dexamethasone for binding to nuclear receptor, with $K_I = 8.1 \times 10^{-6}$ M. Scatchard plot analysis of dexamethasone-receptor binding in the presence or absence of 5′-deoxypyridoxal was consistent with an apparent reduced affinity of [^{3}H]dexamethasone for receptor, which again suggests competitive interaction or allosteric interaction mediated dissociation. Glucocorticoids are known to stimulate alkaline phosphatase activity within HeLa S_3 cells. In whole cell incubations, 5′-deoxypyridoxal was effective in reducing the dexamethasone-induced increase in alkaline phosphatase activity by 60% under conditions in which cell viability and cell growth were not affected.

Pyridoxal 5′-phosphate, the coenzymatically active form of vitamin B-6, is known to alter the molecular properties of several steroid hormone receptors, including the rat uterine estrogen receptor (Muldoon & Cidlowski, 1980), rat thymus (Cidlowski & Thanassi, 1979), HeLa S_3 glucocorticoid receptor (O'Brien & Cidlowski, 1981), and avian progesterone receptor (Nishigori & Toft, 1979). It appears that pyridoxal phosphate reacts with an amino group of a specific lysine residue(s) in a covalent Schiff-base linkage. 5′-Deoxypyridoxal, a vitamin B-6 analogue, also appears to affect the properties of steroid hormone receptors (O'Brien et al., 1980). In preliminary studies it was reported that millimolar concentrations of 5′-deoxypyridoxal caused a loss of [^{3}H]dexa-

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methasone binding to receptor complexes in whole cell incubations from rat thymus and HeLa S_3 cells (O'Brien et al., 1980). These studies have been extended in an attempt to understand the mechanism(s) by which 5′-deoxypyridoxal alters glucocorticoid binding to receptor. In this report, 5′-deoxypyridoxal is shown to be competitive with dexamethasone for receptor binding and to increase the dissociation of prebound dexamethasone from receptor in a pH-dependent fashion. This effect is highly specific for 5′-deoxypyridoxal and is reversible. Furthermore, this inhibition of binding to receptor is effective in reducing the dexamethasone-induced increase in alkaline phosphatase activity in HeLa S_3 cells. These data suggest that 5′-deoxypyridoxal may be a useful compound to gain information regarding the nature of the steroid binding site on the glucocorticoid receptor.

Experimental Procedures

Materials. [6,7-$^3H_2$]Dexamethasone (36.0 Ci/mmol) was purchased from New England Nuclear and stored at 4 °C in benzene/ethanol (9:1 v/v). Aliquots were placed in polyethylene Eppendorf tubes or 25-mL Erlenmeyer flasks, and the solvent was evaporated prior to the addition of cell suspensions. Stock solutions of unlabeled dexamethasone (Steraloids) were prepared at a concentration of 1×10^{-4} M in Krebs–Ringer bicarbonate buffer with 10 mM glucose (KRBG).[1] 5′-Deoxypyridoxal was prepared by the method of Muhlradt & Snell (1967) by Dr. John W. Thanassi, who kindly provided us with this compound. 5′-Deoxypyridoxamine and 5′-deoxypyridoxine were prepared by catalytic hydrogenation of the phosphorylated precursors (Cunningham & Thanassi, 1979). Pyridoxal 5′-phosphate, pyridoxine, and pyridoxamine 5′-phosphate were purchased from Aldrich Chemical Co.; pyridoxal and pyridoxamine were obtained from Sigma Chemical Co. Stock solutions of vitamin B-6 compounds and analogues (50 mM) were prepared in 1.5 mM $MgCl_2$, adjusted to the desired pH with 1 N KOH, and frozen until use. Dextran-coated charcoal (1.0% Norit A, 0.1% dextran) was prepared in 1.5 mM $MgCl_2$. *p*-Nitrophenyl phosphate, *p*-nitrophenol, and 221 alkaline buffer solution (2-amino-2-methyl-1-propanol) were also purchased from Sigma Chemical Co. All other chemicals were the best grade available from Fisher Scientific Co., Baker Chemical Co., or Sigma Chemical Co.

Methods. HeLa S_3 cells were obtained from Microbiological Associates and grown as monolayers in Joklik's medium containing 75 units/mL penicillin G and 50 units/mL streptomycin sulfate (GIBCO). The medium was supplemented with 2 mM glutamine and 5% heat-inactivated fetal calf serum as previously described (O'Brien & Cidlowski, 1981). The cells were harvested by treatment with Versene (GIBCO) and washed twice with unsupplemented Joklik's medium, and the cell pellet was resuspended to a final concentration of (1–3) $\times 10^7$ viable cells/mL. The cell viability was determined in a hemacytometer with exclusion of 0.4% trypan blue as a criterion for life. The cell number was obtained on a Model ZF Coulter counter. Cell viability was routinely greater than 90%.

Glucocorticoid Receptor Binding Assays. (*A*) *Cytoplasmic Assay.* Whole cells were suspended in unsupplemented Joklik's medium for 1.5–2 h with [^{3}H]dexamethasone (2×10^{-8} M or as indicated) in the presence and absence of a 100-fold molar excess of unlabeled dexamethasone. Cytoplasmic fractions were prepared by homogenization followed by centrifugation at 10000*g*, and [^{3}H]dexamethasone–receptor complexes were quantitated in aliquots of cytosol by using a dextran-coated charcoal adsorption method (O'Brien & Cidlowski, 1981). In brief, aliquots (40–50 μL) of cytosol were sampled into 0.10-mL of a dextran-coated charcoal suspension and incubated for 10 min at 2 °C. The charcoal was removed by a 1-min centrifugation at 10000*g*, and aliquots of the supernatant (80–100 μL) were placed in 5-mL scintillation vials (Walter Sarstedt Co.). Three milliliters of phase combining system scintillation fluid (Amersham)/xylenes (Baker) (2:1 v/v) was added, and the sample radioactivity was determined in a Beckman LS-250 scintillation spectrometer having a 40% efficiency for tritium. No significant variation among the samples counted was observed, allowing for the use of raw data.

(*B*) *Nuclear Assay.* HeLa S_3 nuclear receptor binding was measured by the freeze–thaw hypotonic lysis procedure previously described (Littlefield et al., 1980).

pH Dependence of Loss of Dexamethasone-Receptor Binding. Cytosol was prepared in Tris buffer, adjusted to pH 5.8, 6.3, 6.8, 7.3, 7.6, 7.8, or 8.8. Citrate (5 mM) was added to buffers of pH less than 6.8 to allow adequate buffering capacity. Cytoplasmic [^{3}H]dexamethasone receptors were assayed as described above.

Treatment of Dexamethasone–Receptor Complexes with Vitamin B-6 Compounds and Analogues. A 10 times concentrated solution of pyridoxal phosphate, 5′-deoxypyridoxal, or other vitamin B-6 analogues was prepared as described under Experimental Procedures. A 10 times concentrated solution was added to a whole cell suspension or cytosol preparation in a 1:10 dilution to give the desired final concentration of compound.

Titration of 5′-Deoxypyridoxal with 1.00 N NaOH. 5′-Deoxypyridoxal was dissolved in double-distilled water acidified with (1.0 N) HCl to a concentration of 6.25 mM. NaOH (1.00 N) was added in 5-μL aliquots, and the pH was measured after the solution was allowed to be stirred for 1 min.

Determination of Alkaline Phosphatase Activity. Alkaline phosphatase activity was determined as previously described (Littlefield et al., 1980). HeLa S_3 cells were harvested at concentrations of 8×10^6 to 4×10^7 cells/mL and frozen until analyzed for enzyme activity. The frozen cells were thawed and refrozen and thawed to lyse the cells, and 50-μL aliquots were incubated in reaction mixtures containing 6.8 mM *p*-nitrophenyl phosphate and 0.68 M Sigma 221 AMP buffer (2-amino-2-methyl-1-propanol), pH 10.3. The total incubation volume (0.5 mL) was kept at 37 °C for 15 min. The reaction was stopped by the addition of 10 volumes of 0.05 N NaOH. *p*-Nitrophenol production was monitored by absorption at 400 nM in a Beckman Model 24 spectrophotometer. One unit of enzyme activity is defined as 1 μmol of *p*-nitrophenol production/h at 37 °C.

Sucrose Density Gradient Centrifugation. Sucrose gradients (5–20%) were prepared and centrifuged as previously indicated (O'Brien & Cidlowski, 1981).

Scatchard Analysis of Receptor Binding. A series of incubation tubes were prepared containing a range of [^{3}H]-dexamethasone concentrations from 9×10^{-10} to 2×10^{-6} M plus or minus 50 μM 5′-deoxypyridoxal. The cell suspension was added to a total volume of 0.2 mL. The atmosphere was flushed with 5% CO_2/95% air, and the cell suspensions were incubated at 37 °C for 1 h. Duplicate 75-μL aliquots were removed from each incubation and nuclear dexamethasone

[1] Abbreviations: KRBG, Krebs–Ringer bicarbonate with glucose; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; dex, dexamethasone (9α-fluoro-11β,17,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione).

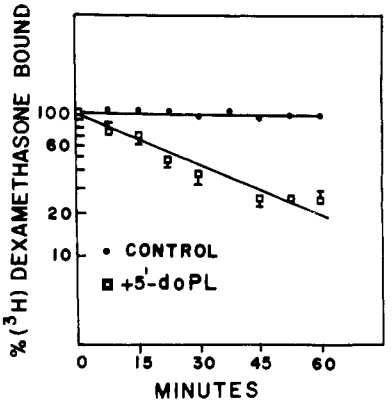

FIGURE 1: Loss of cytosolic [^{3}H]dexamethasone-receptor binding in the presence of 5 mM 5'-deoxypyridoxal. Cytosol was prepared in Tris buffer, pH 7.8, and incubated in the presence (□) or absence (●) of 5 mM 5'-deoxypyridoxal. Duplicate aliquots were assayed for cytoplasmic [^{3}H]dexamethasone binding at time intervals up to 60 min. The difference in binding values obtained with and without unlabeled dexamethasone represents the saturable receptor fraction. Data are plotted as percent [^{3}H]dexamethasone bound and are corrected for loss that occurs in the absence of 5'-deoxypyridoxal.

receptors quantitated (Littlefield et al., 1980). Unbound [^{3}H]dexamethasone was determined by sampling 25 μL of cell suspension from each dilution, centrifuging (10000*g*, 1 min), and sampling the supernatant to determine radiolabel concentration. The data were analyzed according to Scatchard (1949) after graphical correction (Rosenthal, 1967).

Results

Influence of 5'-Deoxypyridoxal on Isolated Cytoplasmic Glucocorticoid Receptors. It was previously demonstrated that pyridoxal phosphate can affect the sedimentation coefficient of HeLa S_3 cytoplasmic dexamethasone receptors without any significant alteration in the quantity of receptor binding (O'Brien & Cidlowski, 1981). This effect was specific and not observed for any other vitamin B-6 analogue. However, inclusion of the analogue 5'-deoxypyridoxal during sucrose gradient analysis of the receptor was observed to significantly reduce the binding of [^{3}H]dexamethasone to the receptor. On the basis of these preliminary observations (O'Brien & Cidlowski, 1981), we initiated a series of experiments to study the nature of the effect of 5'-deoxypyridoxal on the glucocorticoid receptor. The time-dependent effect of 5'-deoxypyridoxal on the loss of [^{3}H]dexamethasone binding to isolated cytoplasmic receptor is shown in Figure 1. Cytoplasmic preparations of [^{3}H]dexamethasone were incubated for 60 min at 2 °C in the presence of 5 mM 5'-deoxypyridoxal. Aliquots were removed at 5-min intervals, and binding was quantitated by the charcoal adsorption technique. A first-order exponential decrease in bound [^{3}H]dexamethasone was observed, with $K_{diss} = 1.70 \times 10^{-2}$ min^{-1}. The half-life of the loss of [^{3}H]dexamethasone binding in the presence of 5'-deoxypyridoxal was $t_{1/2} = 40$ min. The percent bound [^{3}H)dexamethasone was corrected for the loss of [^{3}H]dexamethasone binding that occurs in the absence of added 5'-deoxypyridoxal. After 60 min, the loss of [^{3}H]-dexamethasone binding in the absence of added 5'-deoxypyridoxal represented only 4% of the total initial binding in control HeLa S_3 cytosol ($K_{diss} = 6.0 \times 10^{-4}$ min^{-1}). The dissociation rate of bound [^{3}H]dexamethasone from receptor complexes in the presence of 5'-deoxypyridoxal is approximately 70-fold greater than that found in control cytosol.

The rate of loss of dexamethasone-receptor binding in the presence of 5'-deoxypyridoxal was pH dependent (Figure 2). Cytoplasmic [^{3}H]dexamethasone–receptor complexes were incubated in the presence of 5'-deoxypyridoxal at 5 mM

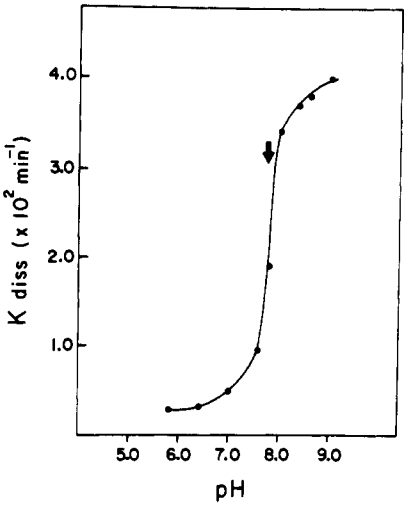

FIGURE 2: pH dependence of loss of [^{3}H]dexamethasone-receptor binding in the presence of 5'-deoxypyridoxal. Cytosol aliquots of 0.1 mL were diluted with 1.9 mL of Tris buffer adjusted to pH 5.8–9.0 and homogenized as described under Experimental Procedures. 5'-Deoxypyridoxal was added to 5 mM, and duplicate aliquots were removed every 5 min. Cytosol was assayed for cytoplasmic [^{3}H]-dexamethasone binding and corrected for nonsaturable binding. Data were subjected to linear regression analysis, and K_{diss} was determined from the slope. Individual values of K_{diss} are plotted as a function of pH.

concentration for 60 min in Tris buffer, over the pH range of 5.8–8.8. The rate of loss of [^{3}H]dexamethasone-receptor binding was plotted as a function of time. The resultant graphs showed first-order loss of [^{3}H]dexamethasone binding similar to that shown in Figure 1. Rate constants of dissociation (K_{diss}) were calculated from the straight line obtained from linear regression analysis for each graph. Figure 2 shows the plot of K_{diss} vs. pH. The pH dependence of the dissociation of [^{3}H]dexamethasone from cytoplasmic receptor complexes fits a simple sigmoidal titration curve, with an inflection point at pH ~7.8. A 20-fold increase in loss of [^{3}H]dexamethasone binding was observed from pH 5.8 to pH 8.8 (1.81×10^{-3} min^{-1} and 3.86×10^{-1} min^{-1}, respectively). At pH 8.8, K_{diss} is 400-fold increased above that of control preparations incubated in the absence of 5 mM 5'-deoxypyridoxal. A half-life of $t_{1/2} = 21.5$ min at pH 8.8 compares with $t_{1/2} = 1.2 \times 10^3$ min for untreated control dexamethasone–receptor complexes at this pH.

These data suggested the effect of a titratable functional group on the rate of dissociation of [^{3}H]dexamethasone from dexamethasone–receptor complexes. A pK_a of 7.8 is slightly lower than that reported by Metzler and Snell for the pyridinium proton of 5'-deoxypyridoxal (8.14) determined from spectral analysis (Metzler & Snell, 1955). A direct titration of acidified 5'-deoxypyridoxal with NaOH was performed to coompare with pK_a = 8.14 reported for spectral analysis. Equivalents of NaOH were added at intervals to a solution containing 6.25 mM 5'-deoxypyridoxal. A computer analysis of the second derivative of each pH value indicated two inflection points for the titration of 5'-deoxypyridoxal, at pH 3.82 and 7.94 (Figure 3). These pK_a values are in reasonable agreement with those reported for spectral analysis. The titration value for the pyridinium proton (pK_a = 7.94) suggests that this ionizable group may account for the pH dependence of the loss of dexamethasone-receptor binding in the presence of 5'-deoxypyridoxal.

Alternatively, it is possible that titration of a functional group on the receptor protein is responsible for the pH dependence of the 5'-deoxypyridoxal effect on dexamethasone-

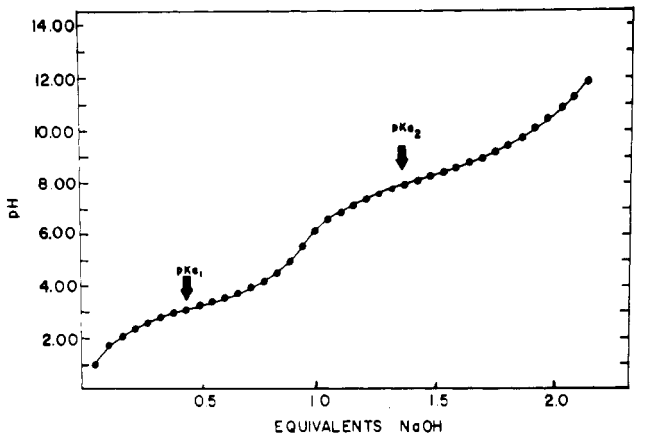

FIGURE 3: Titration of 5′-deoxypyridoxal with 1.00 N NaOH. 5′-Deoxypyridoxal was dissolved in double-distilled water to a concentration of 6.25 mM and acidified with HCl. Addition of 1.00 N NaOH was made in 5-μL aliquots and the pH measured to three significant figures on an Orion Research 701A ionalyzer. The pH of the 5′-deoxypyridoxal solution determined at each addition was plotted vs. equivalents of NaOH.

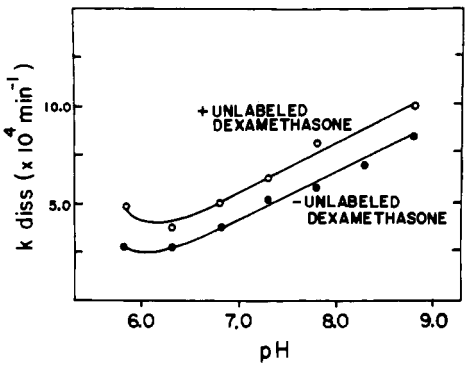

FIGURE 4: pH dependence of loss of [^{3}H]dexamethasone-receptor binding in the presence and absence of unlabeled dexamethasone. Cytosol was prepared in Tris buffer, pH 5.8–8.8, and incubated in the presence and absence of a 100-fold excess of unlabeled dexamethasone for 90 min at 2 °C. Duplicate aliquots were removed every 15 min, and cytoplasmic [^{3}H]dexamethasone binding was determined. Data were corrected for nonsaturable binding and subjected to linear regression analysis. Dissociation constants (K_{diss}) were calculated and plotted as in Figure 3.

receptor binding. That is, an ionizable functional group present at the site of 5′-deoxypyridoxal interaction or a group not directly involved but affecting the interaction by a conformational alteration in the receptor molecule might also produce the observed pH dependence of the 5′-deoxypyridoxal effect on loss of dexamethasone-receptor binding. The likelihood of the second possibility was excluded by showing the direct competition of 5′-deoxypyridoxal and dexamethasone for a protein binding site. The possibility of an ionizable group present at the site of 5′-deoxypyridoxal and dexamethasone binding was directly tested by a determination of the kinetics of dissociation of [^{3}H]dexamethasone from the receptor complex in the presence of excess unlabeled dexamethasone. If the titratable group represents a protein residue affecting the binding of 5′-deoxypyridoxal and dexamethasone, a similar sigmoidal curve of K_{diss} vs. pH should be observed for the dissociation of [^{3}H]dexamethasone from [^{3}H]dexamethasone–receptor complexes in the presence of excess competing unlabeled dexamethasone. Figure 4 represents the effect of pH on the dissociation of [^{3}H]dexamethasone from [^{3}H]dexamethasone–receptor complexes in the presence of 10^{-6} M unlabeled dexamethasone. The effect of pH on the rate of dissociation in the absence of unlabeled dexamethasone is

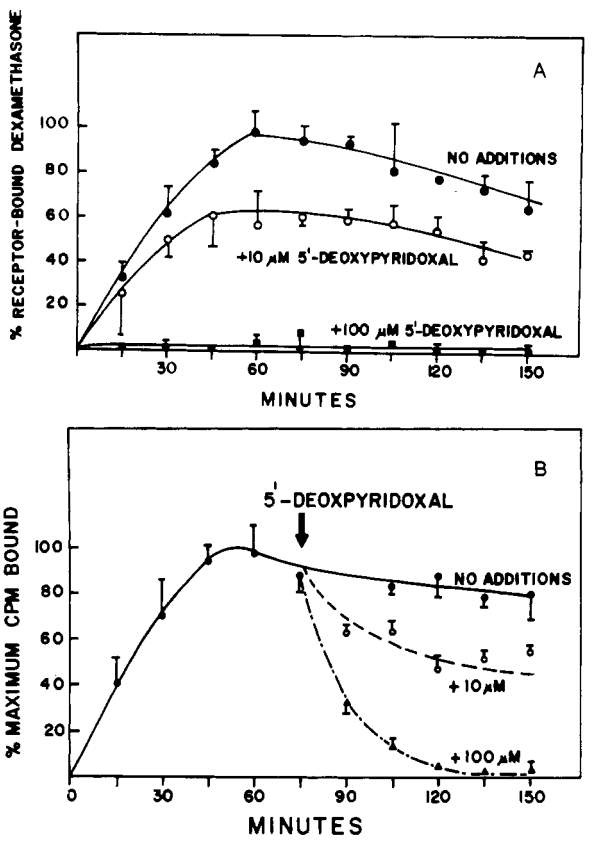

FIGURE 5: (A) Effect of 5′-deoxypyridoxal on accumulation of nuclear [^{3}H]dexamethasone–receptor complexes in whole HeLa S_3 cells at 37 °C. HeLa S_3 cells were suspended in Joklik's medium in a 25-mL Erlenmeyer flask at 37 °C. 5′-Deoxypyridoxal was added to a final concentration of 10 μM (○) or 100 μM (■). Control incubations (●) received and equivalent volume of Joklik's medium. [^{3}H]Dexamethasone was also added to 2.5×10^{-8} M. Triplicate aliquots were removed, and nuclear binding was measured by the hypotonic lysis assay described under Experimental Procedures. The data shown represent the saturable binding fraction (three determinations) averaged from four separate experiments. Nuclear [^{3}H]dexamethasone binding is plotted as the percent maximum receptor-bound [^{3}H]-dexamethasone. Cell viability (95%) was not decreased after a 150-min incubation. (B) Effect of 5′-deoxypyridoxal on loss of nuclear [^{3}H]dexamethasone-receptor binding. HeLa S_3 cells were incubated with [^{3}H]dexamethasone (2.5×10^{-8} M) as in (A), and 5′-deoxypyridoxal was added after 75 min. Data were determined and plotted as in (A).

also shown. Similar curves were obtained for [^{3}H]dexamethasone complexes incubated in the presence and absence of unlabeled dexamethasone. An increase or decrease in buffer pH resulted in a progressive increase in the dissociation rate. No evidence of a titratable functional group was apparent. The values of K_{diss} for dexamethasone receptor are considerably less than those observed in the presence of 5 mM 5′-deoxypyridoxal at higher pH (6.0×10^{-4} min^{-1} vs. 4.0×10^{-2} min^{-1}, pH 8–9). This suggests that loss of dexamethasone-receptor binding in the absence of 5′-deoxypyridoxal either does not involve the same ionizable receptor group or this ionizable group is unique to 5′-deoxypyridoxal, presumably the pyridinium proton.

Influence of 5′-Deoxypyridoxal on Glucocorticoid Receptor in Whole Cells. The effect of 5′-deoxypyridoxal on dexamethasone–receptor complexes was next observed in whole cell incubations. The accumulation of [^{3}H]dexamethasone receptor in 37 °C incubations reached a maximum after 45–60 min (Figure 5A). Under the assay conditions utilized to measure dexamethasone receptor, i.e., 2.5×10^{-8} M dexamethasone, approximately 50% of cellular-associated receptors

Table I: Specificity of 5'-Deoxypyridoxal Effect of Accumulation of Nuclear Dexamethasone Receptor in Whole HeLa S_3 Cells at 37 °C[a]

compound (10^{-4} M)	percent of control cpm
pyridoxal 5'-phosphate	110.1 ± 6.5
pyridoxal	95.7 ± 5.1
pyridoxine	85.1 ± 13.3
pyridoxamine	99.5 ± 4.6
pyridoxamine 5'-phosphate	99.3 ± 19.0
5'-deoxypyridoxal	3.3 ± 2.1
5'-deoxypyridoxine	110.0 ± 6.5
5'-deoxypyridoxamine	95.6 ± 7.8

[a] HeLa S_3 cells were harvested and suspended in unsupplemented Joklik's medium. Cells were incubated at 37 °C in the presence of 2.0×10^{-8} M [^{3}H]dexamethasone with or without vitamin B-6 analogues. Under these assay conditions approximately 35% of cellular receptors are occupied by exogenously added tritiated ligand. At 60 min, duplicate aliquots were sampled, and nuclear binding was assayed by the hypotonic lysis method. A control incubation was performed in the absence of added vitamin B-6 analogue. Identical volume was maintained in the control by the addition of Joklik's medium to the same volume as the experimental incubations. The data shown represent the saturable binding fraction (mean ± SD, two determinations) calculated by subtracting binding values obtained in the presence of a 100-fold excess of unlabeled dexamethasone.

Table II: Reversibility of Effect of 5'-Deoxypyridoxal on [^{3}H]Dexamethasone-Receptor Binding in Whole HeLa S_3 Cells[a]

addition	cpm of bound [^{3}H]dexamethasone (±SD, four determinations) at 60 min	120 min
none	795.4 ± 141.3	715.3 ± 178.8
5'-deoxypyridoxal (0.1 M)	118.8 ± 17.9	648.6 ± 12.3

[a] HeLa S_3 cells were harvested and resuspended in 5 mL of unsupplemented Joklik's medium. Cell concentration was 1.7×10^7 viable cells/mL (97% viability). Cell suspensions were placed in 12-mL Sarstedt tubes with 1.8×10^{-8} M [^{3}H]dexamethasone in the presence and absence of 1×10^{-4} M 5'-deoxypyridoxal. The atmosphere was flushed with 5% CO_2/95% air and incubated at 37 °C for 60 min with constant agitation. Duplicate 40-μL aliquots were sampled at 1 h from each tube into 1.0 mL of 1.5 mM $MgCl_2$ and assayed for nuclear [^{3}H]dexamethasone binding by the hypotonic lysis assay. Additional duplicate 40-μL aliquots were diluted with 2.0 mL of unsupplemented Joklik's medium containing 1.8×10^{-8} M [^{3}H]dexamethasone in the presence and absence of 1.8×10^{-6} M unlabeled dexamethasone. The cytosols were incubated for 60 min and the cells pelleted by centrifugation (800 *g*, 5 min). The cell pellet was resuspended in 1.0 mL of 1.5 mM $MgCl_2$ and assayed for nuclear [^{3}H]dexamethasone-receptor binding by the hypotonic lysis assay described under Experimental Procedures.

are occupied by the exogenously added ligand. Subsequently, the amount of nuclear receptors decreased to 75% maximum by 150 min. In the presence of 1 μM 5'-deoxypyridoxal, nuclear dexamethasone-receptor concentration reached a maximum of 60% that of the control incubations by 45–60 min. A similar decrease in binding paralleling that observed for control dexamethasone receptors resulted in only 40% of the maximum control binding observed by 150 min. At 100 μM 5'-deoxypyridoxal, nuclear receptor accumulation is reduced to less than 4% that of the maximum control value.

Addition of 5'-deoxypyridoxal to nuclear receptor complexes after preincubation with [^{3}H]dexamethasone also caused the loss of nuclear dexamethasone-receptor binding at 37 °C (Figure 5B). Whole cell suspensions were incubated in the presence of [^{3}H]dexamethasone for 75 min, at which time 5'-deoxypyridoxal was added. At 150 min in the presence of 10 μM 5'-deoxypyridoxal, 45% of the maximum control-bound [^{3}H]dexamethasone remained, a similar decrease as that found after 150 min of incubation in the presence of 5'-deoxypyridoxal (Figure 5A). Addition of 100 μM 5'-deoxypyridoxal at 75 min resulted in a reduction of bound [^{3}H]dexamethasone to less than 4% of the maximum control-bound [^{3}H]dexamethasone by 150 min. Thus, whether the 5'-deoxypyridoxal incubation was performed for 75 min or 150 min, the amount of nuclear dexamethasone receptor present at 150 min was comparable.

The effectiveness of 5'-deoxypyridoxal in decreasing the nuclear concentration of dexamethasone receptor was specific and not observed for other vitamin B-6 analogues tested. Eight vitamin B-6 analogues were added to separate HeLa S_3 cell suspensions at a final concentration of 100 μM and incubated for 60 min at 37 °C. The results in Table I show that in cells incubated in the presence of 100 μM 5'-deoxypyridoxal accumulated only 3.5% of those nuclear dexamethasone–receptor complexes found for untreated cells. No significant effect on the amount of saturable [^{3}H]dexamethasone was observed for the other analogues tested. Only pyridoxine was effective in reducing the amount of [^{3}H]dexamethasone binding to 85.1 ± 13.3%. This small effect is probably the result of experimental error and was not observed in other pyridoxine incubations.

The reversibility of the effect of 5'-deoxypyridoxal on nuclear dexamethasone receptor was next examined. HeLa S_3 cells were incubated in the presence of 100 μM 5'-deoxypyridoxal for 60 min. Nuclear receptor binding was assayed, and the remaining cells were diluted 1:50 in a medium containing [^{3}H]dexamethasone but without 5'-deoxypyridoxal. Nuclear binding was assayed again at 120 min (Table II). After 60 min, control [^{3}H]dexamethasone binding in HeLa S_3 cell nuclei measured 795.4 cpm/assay. After a total of 120 min, this level of binding was reduced by 9.8% to 715.3 cpm. In the presence of 5'-deoxypyridoxal, nuclear dexamethasone-receptor binding was significantly less than that observed in control samples, reaching only 118.8 cpm, or 16.6% that of control incubation after 60 min. However, a 1:50 dilution of 5'-deoxypyridoxal and a further incubation in the presence of [^{3}H]dexamethasone for an additional 60 min increased [^{3}H]dexamethasone binding significantly (648.6 cpm). This represents 90.7% of that found for control samples at 120 min, incubated in the absence of 5'-deoxypyridoxal. This reversal is not 100% complete, as 2 μM 5'-deoxypyridoxal is still effective in inhibiting nuclear dexamethasone-receptor binding (data not shown).

The effect of 5'-deoxypyridoxal on the [^{3}H]dexamethasone–receptor complex was also characterized as competitive with dexamethasone for receptor binding. Cells were incubated with varying concentrations of dexamethasone in the presence of 0, 1.0×10^{-5}, 2.0×10^{-5}, or 5.0×10^{-5} M 5'-deoxypyridoxal at 37 °C, and after 60 min, nuclear dexamethasone-receptor binding was determined. A double-reciprocal plot analysis of 1/(bound cpm) vs. 1/([^{3}H]dexamethasone concentration) yielded a common *y* intercept for each plot generated at varying concentrations of 5'-deoxypyridoxal, thus indicating the competitive nature of the 5'-deoxypyridoxal on binding (data not shown). A linear replot of the individual slopes determined from the double-reciprocal plot vs. [^{3}H]dexamethasone concentration also yielded a straight line with an *x*-axis intercept of 8.1×10^{-6} M. Thus, the competition exhibited by 5'-deoxypyridoxal is competitive, with a $K_I = 8.1 \times 10^{-6}$ M (data not shown).

The dexamethasone binding properties of nuclear dexamethasone receptor in the presence of 5'-deoxypyridoxal were

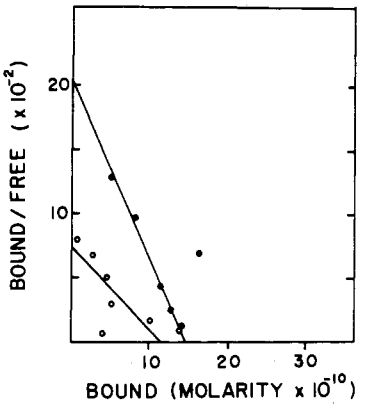

FIGURE 6: Scatchard analysis of nuclear [^{3}H]dexamethasone receptor in the presence of 5′-deoxypyridoxal. A series of incubation tubes were prepared containing 9×10^{-10} to 2×10^{-6} M [^{3}H]dexamethasone plus or minus 50 μM 5′-deoxypyridoxal. The cell suspension was added, the atmosphere was flushed with 5% CO_2/95% air, and the cells were incubated at 37 °C for 1 h. The concentration of bound [^{3}H]dexamethasone was determined by nuclear binding assay. Each point represents the average of duplicate determinations. Control cells (●) had a dissociation constant of 7.2×10^{-9} M, and 5′-deoxypyridoxal-(○) treated cells had a dissociation constant of 2.3×10^{-8} M.

compared to control receptors by Scatchard analysis (Figure 6). A concentration of 50 μM 5′-deoxypyridoxal was chosen to allow an observable effect of 5′-deoxypyridoxal yet still allow binding of [^{3}H]dexamethasone to receptor. The equilibrium constant, K_D, determined from the slope of each line, was significantly different for dexamethasone receptor incubated in the presence of 50 μM 5′-deoxypyridoxal. The equilibrium constant of control dexamethasone receptor was $K_D = 7.2 \times 10^{-9}$ M. The equilibrium constant of 5′-deoxypyridoxal-treated dexamethasone receptor was significantly higher at $K_D = 2.3 \times 10^{-8}$ M, supporting the competitive nature of 5′-deoxypyridoxal and [^{3}H]dexamethasone with nuclear dexamethasone receptor.

5′-Deoxypyridoxal Inhibition of Dexamethasone Induction of Alkaline Phosphatase Activity. The above results indicated a competitive, reversible action of 5′-deoxypyridoxal with dexamethasone for receptor binding. This encouraged us to extend these studies to determine if a known physiological effect of dexamethasone, presumably mediated through steroid hormone receptor, could be blocked or inhibited in part by 5′-deoxypyridoxal in addition to whole cells. In the presence of hydroxycortisone, an active glucocorticoid hormone, a 5–20-fold increase in HeLa S_3 cell alkaline phosphatase was orginally described by Cox & Elson (1971) and subsequently shown to result from an increased catalytic efficiency of the induced alkaline phosphatase relative to the base-level (untreated) enzyme (Griffin & Cox, 1966). The dexamethasone-induced increase in alkaline phosphatase activity is a time-dependent and concentration-dependent response. The effect is only observed in cells treated with active glucocorticoid and is not observed in cells grown in the presence of inactive glucocorticoids or different classes or steroid hormones (Littlefield et al., 1980).

Figure 7A shows the effect of 5′-deoxypyridoxal on the dexamethasone-induced increase in HeLa S_3 cell alkaline phosphatase activity. In the presence of 10^{-7} M dexamethasone, a saturating concentration of steroid that occupies all cellular receptors, alkaline phosphatase activity was increased 7-fold over that of control cells after a 3-day incubation. No effect on alkaline phosphatase activity was noticed for cells grown in the presence of 10^{-4} M 5′-deoxypyridoxal. However, in the presence of 10^{-4} M 5′-deoxypyridoxal in addition to 10^{-7} M dexamethasone, the increase in alkaline

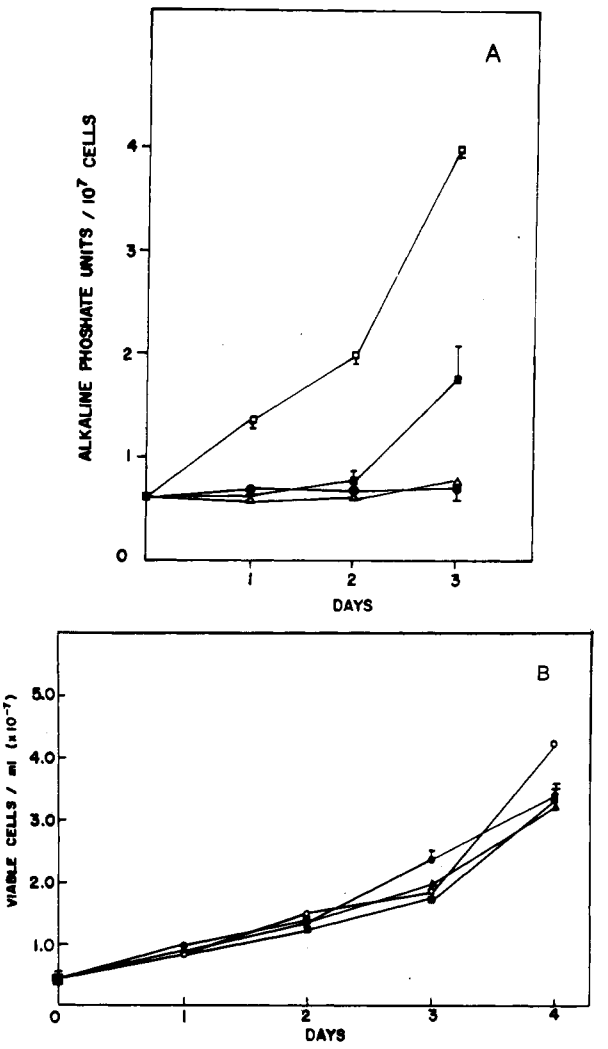

FIGURE 7: (A) Effect of 5′-deoxypyridoxal on dexamethasone-induced increase in HeLa S_3 alkaline phosphatase activity. HeLa S_3 cells were plated at a density of approximately 2×10^6 viable cells/mL and allowed to attach for 24 h. The media was removed, and cells were exposed to medium containing 1×10^{-7} M dexamethasone (□), 1×10^{-4} M 5′-deoxypyridoxal (●), 1×10^{-7} M dexamethasone plus 1×10^4 M 5′-deoxypyridoxal (■), or no addition (△). The medium was replaced with fresh medium on day 2. Cells were harvested on days 0, 1, 2, and 3 after addition and viability and cell number determined. Cells were stored frozen for assay of alkaline phosphatase activity after 3 days. Alkaline phosphatase activity was determined as described under Experimental Procedures. (B) Effect of 5′-deoxypyridoxal and dexamethasone on cell growth. Cell numbers were determined on a Coulter counter, and viability was determined by trypan blue exclusion as a criterion for life. Cell viability was in excess of 95% in all treatment groups: control cells (●); 10^{-4} M 5′-deoxypyridoxal (■); 10^{-7} M dexamethasone (○), 10^{-7} M dexamethasone plus 10^{-4} M 5′-deoxypyridoxal (△).

phosphatase activity was considerably less than that found in the presence of dexamethasone alone. The 7-fold increase in alkaline phosphatase activity previously observed in cells grown in the presence of dexamethasone was reduced to a 3-fold increase in alkaline phosphatase activity over control levels. Furthermore, no increase in alkaline phosphatase activity was observed in dexamethasone plus 10^{-4} M 5′-deoxypyridoxal cultured cells after 1 and 2 days, although alkaline phosphatase activity of dexamethasone alone cultured cells increased 2-fold and 3-fold, respectively. This inhibition of the dexamethasone-induced increase in alkaline phosphatase activity in the presence of 10^{-4} M 5′-deoxypyridoxal indicated the ability of this vitamin B-6 analogue to inhibit a glucocorticoid effect in cultured cells. That this effect cannot be attributed

to a decrease in cell growth or viability in cells treated with 5′-deoxypyridoxal is shown in Figure 7B.

Cell growth and viability was monitored in the HeLa S_3 cultures grown in the presence and absence of dexamethasone and 5′-deoxypyridoxal (Figure 7B). After 2 days, very little alteration was noticed in cell growth. It appears then that neither 5′-deoxypyridoxal nor dexamethasone significantly influences cell growth or viability (in excess of 90%) after 3 days. 5′-Deoxypyridoxal is thus able to affect a known physiological response of dexamethasone in HeLa S_3 cells in culture, presumably by the direct competition of 5′-deoxypyridoxal with dexamethasone for the site(s) of action.

Discussion

In this paper we have presented data that indicate that the vitamin B-6 analogue 5′-deoxypyridoxal can compete with or displace [^{3}H]dexamethasone from glucocorticoid receptors either in isolated cytosol preparations or in whole cells at 37 °C. This effect was achieved at millimolar concentrations of 5′-deoxypyridoxal at 0–4 °C and was observable at micromolar concentrations when nuclear receptor levels are measured at 37 °C in whole cells. In an attempt to understand the mechansim of 5′-deoxypyridoxal action we have studied the influence of 5′-deoxypyridoxal on the kinetics of glucocorticoid receptor binding. Addition of this vitamin B-6 analogue to isolated, cytoplasmic preparations indicates that 5′-deoxypyridoxal increases K_{diss} for [^{3}H]dexamethasone–receptor interaction. This effect, unlike dissociation of steroid in the absence of 5′-deoxypyridoxal, is highly pH dependent and is probably dependent on the deprotonation of the pyridinium nitrogen present in 5′-deoxypyridoxal.

It appears, on the basis of the data presented, that 5′-deoxypyridoxal competes directly with [^{3}H]dexamethasone for the steroid binding site, or at least in the same region on the protein, or perhaps at a different site on the steroid receptor, which allosterically influences steroid binding. The fit of 5′-deoxypyridoxal in the receptor binding site is not of high affinity as is indicated by the K_I of 8.1×10^{-6} M. On the basis of a competitive interaction for 5′-deoxypyridoxal with receptor, we would predict an apparent decrease in receptor affinity, but little changes in binding site number under Scatchard plot analysis of saturation curve binding data are evaluated in the presence of 5′-deoxypyridoxal. As shown in Figure 6, an apparent decrease in binding affinity is observed with only minimal effects on binding site number. Further evidence for a competitive interaction of 5′-deoxypyridoxal with the dexamethasone receptor is the reversibility of the interaction. A simple 1:50 dilution of cells readily reverses the action of the vitamin B-6 analogue. Finally, the kinetics of loss of prebound nuclear binding of receptor following the addition of 5′-deoxypyridoxal to cells equilibrated with [^{3}H]dexamethasone are quite similar to those we have reported in the past (Cidlowski & Munck, 1980) for an unlabeled dexamethasone of isolated rat thymocytes.

Perhaps the most important evidence for direct interaction between 5′-deoxypyridoxal and glucocorticoid receptor is derived from our studies considering glucocorticoid-induced alkaline phosphatase activity. 5′-Deoxypyridoxal (10^{-4} M) had no effect on HeLa S_3 cell growth, viability, or alkaline phosphatase activity. This concentration of vitamin B-6 analogue was however effective in reducing nuclear [^{3}H]-dexamethasone receptor binding, and inclusion of this concentration of 5′-deoxypyridoxal during growth of HeLa S_3 cells blocks the accumulated action of the steroid on alkaline phosphatase activity. Subsequently, the action of 5′-deoxypyridoxal in blocking the effect of dexamethasone was only partial. Although we can only speculate at this time, perhaps the loss of antagonist action was due to the metabolism of 5′-deoxypyridoxal to a less potent antagonist form.

Although the usefulness of 5′-deoxypyridoxal in steroid receptor biochemistry may at first not appear obvious, several potential uses for 5′-deoxypyridoxal are apparent. First, 5′-deoxypyridoxal is an excellent glucocorticoid antagonist, blocking dexamethasone effects on alkaline phosphatase without any agonist activity or effects on cell growth or cell viability. Second, 5′-deoxypyridoxal may be a useful reversing agent in altering the affinity of steroid–receptor interaction both in whole cells and in isolated cytosol fractions. This may be particularly important in the elution of receptor from steroid affinity columns or in the study of whole cell receptor turnover. Finally, 5′-deoxypyridoxal may be useful in affinity labeling the glucocorticoid receptor at or near the steroid binding site. Each of these notions are currently being entertained.

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