We previously identified vitamin B$_6$ deficiency in a child presenting with seizures whose primary diagnosis was the inherited disorder hyperprolinemia type II. This is an unrecognized association, which was not explained by diet or medication. We hypothesized that pyridoxal phosphate (vitamin B$_6$ coenzyme) was de-activated by 1-$\Delta^1$-pyrroline-5-carboxylic acid, the major intermediate that accumulates endogenously in hyperprolinemia type II. The proposed interaction has now been investigated in vitro with high resolution $^1$H nuclear magnetic resonance spectroscopy and mass spectrometry at a pH of 7.4 and temperature of 310 K. Three novel adducts were identified. These were the result of a Claisen condensation (or Knoevenagel type of reaction) of the activated C-4 carbon of the pyrroline ring with the aldehyde carbon of pyridoxal phosphate. The structures of the adducts were confirmed by a combination of high performance liquid chromatography, nuclear magnetic resonance, and mass spectrometry. This interaction has not been reported before. From preliminary observations, pyrroline-5-carboxylic acid also condenses with other aromatic and aliphatic aldehydes and ketones, and this may be a previously unsuspected generic addition reaction. Pyrroline-5-carboxylic acid is thus found to be a unique endogenous vitamin antagonist. Vitamin B$_6$ de-activation may contribute to seizures in hyperprolinemia type II, which are so far unexplained, but they may be preventable with long term vitamin B$_6$ supplementation.

Vitamin B$_6$ (pyridoxine and related compounds) is distributed widely in food (1–2). Severe nutritional deficiency is not common in humans (3) and has generally arisen in unusual circumstances. Most reports were in the early 1950s and described babies who presented with seizures after being fed with processed milks lacking the vitamin for over 2 months (4). Deficiency has also developed during treatment with drugs prescribed babies who presented with seizures after being fed with processed milks lacking the vitamin for over 2 months (4). Severe nutritional deficiency is not commonly seen in humans (3) and has generally arisen in unusual circumstances. Most reports were in the early 1950s and described babies who presented with seizures after being fed with processed milks lacking the vitamin for over 2 months (4).

The proposed interaction has now been investigated in vitro with high resolution $^1$H nuclear magnetic resonance spectroscopy and mass spectrometry at a pH of 7.4 and temperature of 310 K. Three novel adducts were identified. These were the result of a Claisen condensation (or Knoevenagel type of reaction) of the activated C-4 carbon of the pyrroline ring with the aldehyde carbon of pyridoxal phosphate. The structures of the adducts were confirmed by a combination of high performance liquid chromatography, nuclear magnetic resonance, and mass spectrometry. This interaction has not been reported before. From preliminary observations, pyrroline-5-carboxylic acid also condenses with other aromatic and aliphatic aldehydes and ketones, and this may be a previously unsuspected generic addition reaction. Pyrroline-5-carboxylic acid is thus found to be a unique endogenous vitamin antagonist. Vitamin B$_6$ de-activation may contribute to seizures in hyperprolinemia type II, which are so far unexplained, but they may be preventable with long term vitamin B$_6$ supplementation.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s).
nuclear magnetic resonance (NMR) spectroscopy.

Preliminary NMR and high performance liquid chromatography-mass spectrometry (HPLC-MS) studies confirmed a reaction between PP and P5C co-incubated in aqueous solution at physiological pH (7.4) and temperature (310 K). Three adducts were produced, which we have characterized and found to be novel. They result from a Claisen condensation (or Knoevenagel type of reaction) involving the pyridoxal phosphate carbonyl group and the activated C-4 carbon of the pyrroline ring. This reaction has not been reported before. The most common type of in vivo enzymatic reaction of PP is in transamination, the transfer of an α-amino group of an amino acid to the α-carbon atom of an α-keto acid (5, 11). These reactions are reversible and involve the formation of Schiff bases as intermediates. Therefore, P5C is a unique endogenous pyridoxal phosphate antagonist. If this condensation occurs in vivo, as we propose, individuals with hyperprolinemia type II would have an increased vitamin B₆ requirement and be at risk of symptomatic deficiency. In preliminary observations we find that P5C also reacts with a range of aliphatic aldehydes and ketones and benzaldehyde at physiological pH. This condensation may be a newly recognized general reaction with activated carboxyls.

EXPERIMENTAL PROCEDURES

Materials—DL-Δ¹-Pyrroline-5-carboxylic acid, 2,4-dinitrophenylhydrazine hydrochloride double salt, pyridoxal-5-phosphate, pyridoxamine dihydrochloride, 4-pyridoxic acid, pyridoxine, and pyridoxal hydrochloride were from Sigma, and acetophenone, benzaldehyde, and aliphatic aldehydes were from Aldrich (Sigma-Aldrich Company Ltd., Poole, UK). Deuterium oxide (D₂O), deuterium chloride (DCl), and sodium deuterioxide (NaOD) were from Goss Scientific Instruments Ltd., Great Baddow, UK. Sodium-3-trimethylsilyl-1-[2,2,3,3-2H₄]propionate (TSP) was from Fluorochem Ltd., Old Glossop, Derbyshire, UK, and diethyl ether (Analar) from BDH, Poole, Dorset, UK. Glassware for P5C preparation was acid-washed.

Preparation of P5C and Pyridoxal Phosphate Co-incubates—The P5C was prepared from its 2,4-dinitrophenylhydrazine hydrochloride double salt, using a method based on that of Mezl and Knox (12) but modified by using a more concentrated starting solution of the hydrazine and a diethyl ether wash instead of toluene to remove excess acetophenone. Preparations made according to the published method were found by NMR to be contaminated with toluene and acetophenone, and P5C was polymerized. Thus a slightly modified method was...
adopted. To 40 mg of the hydrazine in a glass tube were added 2.0 ml of 0.25 M DCl and 8 ml (0.2 ml/mg hydrazine) of acetonophene. The mixture was shaken on a roller mixer for 30 min in a cold room at 4 °C. After centrifugation, the lower aqueous layer was transferred to a clean tube and washed with ~8 ml of diethyl ether five times to remove acetonophene. Ether was removed from the aqueous extract by evaporation under nitrogen at room temperature. After adjusting the pH to 7.4 with NaOD, the extract was stored at 4 °C. NMR confirmed P5C to be present, with no evidence of polymerization or contamination with acetonophene. This is the first report of the NMR spectrum of P5C (shown in Fig. 3A). Contrary to expectation (12) we found that P5C was reasonably stable and stored at 4 °C. NMR—Experiments were carried out at 750.3 MHz (1H) and 188.7 MHz (13C) on a Varian INOVA 750 (17.6 T) spectrometer and at 600.13 MHz (1H), 150.9 MHz (13C), and 242.9 MHz (31P) on a Bruker DRX600 (14.1 T) spectrometer. To 500 μl of each sample in a 5-mm NMR tube, 10 μl (10 μg) of 0.1% w/v TSP in D2O was added to enable accurate quantification and act as an internal shift reference. The one-dimensional 1H NMR experiments were carried out under conditions of full relaxation and good digitization to enable reasonable integration for quantification. The 90° pulses used were calibrated after tuning and matching the rf for each sample at the temperatures for which different measurements were taken.

Typically 65,536 data points were acquired over a 16.02-ppm (9614 Hz at 600 MHz) spectral width or 131,072 data points over 16.0 ppm (12,001 Hz at 750 MHz) using a noesypresat (15) solvent suppression pulse sequence. A mixing time of 100 ms, together with 3 s of presaturation and an acquisition time of 3.4 s at 600 MHz or 5.3 s at 750 MHz, was employed. The gradient nOe experiments were done at 750 and 600 MHz using the same respective data tables as above, with mix times of 0.8–1 s and gradient ratios of 35:5:3:2:1 and from 2000 to 8000 transients per experiment. The diffusion experiments (Fig. 4) were acquired as above at 750 MHz with the diffusion time fixed at 0.5 s. The gradient strength was ramped successively from 0.5 to 30.5 gauss/cm in increments of 2 gauss with 16 experiments each of 64 transients acquired with a repetition time of 5 s per transient.

Two-dimensional experiments TOCSY, HMQC, and HMBC were run at 750 MHz and 310 K (nominal, actual temperature was 311.4 K) in phase-sensitive mode, typically 2048 (or 4096 for HMBC) data points acquired over a 13-ppm spectral width in F2 and F1 for the TOCSY (homonuclear experiment), the proton dimension, and 200 ppm in F1 for the carbon dimension. The TOCSY experiment was carried out with 32 transients for each of 256 increments using a spin lock period of 60 ms and a relaxation delay of 1.5 s, during which the HOD signal was pre-saturated to reduce the dynamic range. A total of 256 transients at a pulse repetition rate of 0.5 s per scan for each of 128 increments in the
**HMQC**, and 440 transients (pulse repetition rate equal to 1.5 s) for 256 increments in the HMBC experiment were employed. Globally optimized alternating-phase rectangular decoupling of the carbon frequencies was employed in the HMQC experiment. Cosine window functions were used in the F2 (acquisition dimension) and linear prediction to 1024 complex data points (coefficients optimized for each experiment) with no window function applied in the F1 dimension, prior to Fourier transformation.

**Phosphorus Spectra**—The phosphorus spectra were acquired at 242.9 MHz (14.1 T) with and without proton decoupling \(^1\)H) using 65,536 data points over 50 ppm with 640 transients for both experiments, employing 90° pulses at a pulse repetition time of 4.7 s. An external reference of phosphoric acid was used for calibration and set to 0 ppm. A line broadening of 1 Hz was used in the exponential multiplication window function to increase the apparent signal to noise prior to Fourier transformation.

**Carbon Spectra**—The carbon spectra were acquired with Waltz-16 (123) decoupling of the protons. A 250 ppm spectral width with 131,072 data points and 102,000 transients (51 h) at a repetition rate per scan of 1.8 s yielded a low signal-to-noise spectrum at 150 MHz. The sample was concentrated to 160 µl of volume and run on a 3-mm dual \(^{13}\)C/\(^1\)H probe at 188.69 MHz. After 40,000 transients at 310 K (nominal, actual temperature was 311.8 K) and a pulse repetition time of 2 s, the spectrum shown as the F1 projection trace in Fig. 5 was obtained.

**HPLC-NMR**—With gradient elution, HPLC-NMR was carried out using a Bruker LC-22 HPLC system. The mobile phase was pumped at 1 ml/min through a C\(_18\) reverse phase column (Phenomenex 250 × 4.6 mm) at 25 °C. The eluents were D\(_2\)O and CH\(_3\)CN (ACN) each with 0.05% trifluoroacetic acid added, starting with 100% D\(_2\)O decreasing to 60% D\(_2\)O and 40% ACN over 25 min, followed by a 2-min 100% ACN purge flush before returning to 100% D\(_2\)O. The eluting fractions were detected by UV (Biosch Lambda 1000) at 254 nm, and the absorption was used as a delayed trigger to stop the pump when the fraction had flowed into the NMR probe cell. The \(^1\)H NMR at 600 MHz was measured while the flow was stopped.

The pulse sequence was noesypresat with double-solvent suppression and ACN \(^{13}\)C satellite decoupling. A selective-shaped rf pulse was employed, with frequency adjusted to the ACN at the center of the spectrum (2 ppm also used as internal reference) and to the residual HOD signal, which reduced its shift value with increasing ACN concentration during the gradient elution. Shape selective gradient shimming of the ACN signal was used to maximize the magnetic field homogeneity. The number of transients taken varied from 128 to 1024 for the different fractions, prior to continuing the HPLC pump and moving on to the next peak in the UV. The number of scans depended upon the fraction’s concentration and the signal-to-noise ratio achieved between 5 and 11 ppm. Each fraction was collected post-NMR using a Foxxy Junior fraction collector and was then run on a Micromass Platform Q-TOF in positive ion electron impact conditions for accurate mass measurement. Erythromycin was co-injected, and the ion at \(m/z = 734.4690\) used as the lock reference mass.

**RESULTS**

The initial analysis was by MS of the mixture, arising from a 24-h incubation at 310 K of 1 ml of P5C and 1 ml of PP solution (Fig. 2). Three new quasimolecular ions (M + H) \(^{-}\) were observed at \(m/z = 297, 341,\) and 359 Da by negative ion electrospray, in addition to the expected quasimolecular ions for P5C at \(m/z = 112\) and PP at \(m/z = 246\) Da. The initial \(^1\)H NMR spectrum at 600 MHz (Fig. 3C, after 36 h of reaction) of the same solution at 298 K showed several products in addition to the two starting components.

In an attempt to analyze these components in situ, while the reaction was still ensuing, a series of diffusion (13) experiments were conducted at 750 MHz for maximum shift dispersion. The mixture was partially resolved into its component parts, termed Diffusion Ordered Spectroscopy (DOSY) (16, 17) as illustrated in Fig. 4. The technique relies upon the individual species having different diffusion coefficients, which often vary

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**Fig. 4. DOSY two-dimensional representation of the spectra, with the vertical diffusion axis distinguishing the various components and the full mixture after 48 h, one-dimensional \(^1\)H NMR at 750 MHz water presaturated spectrum is plotted above for comparison.**
with the size of the molecule. Hence, for neutral compounds, a separation of each compound's spectrum along the diffusion axis occurs with increasing molecular mass. However, if there is molecular association of any kind, the diffusion coefficient of the associated components will change and the above progression no longer holds. The products were obviously the result of

**Fig. 5.** HMOC (blue) and HMBE (red) spectra of the mixture after 12 days, at 310 K, and 750 MHz. The F2 trace is the proton spectrum at 750 MHz; the F1 trace is the $^{13}$C spectrum at 188.8 MHz.
reaction of P5C and PP, because signals with similar shifts and couplings were observed. The question was, what structures did they have?

The lack of any additional aldehyde proton signals around 9.5–10.5 ppm indicated that reaction had occurred at that site on the PP. The appearance of a finely coupled triplet of doublets at δ 6.93 suggested an olefinic proton with three remote coupling partners. A series of one-dimensional TOCSY experiments established that these were a pair of double, double doublets (ddd) at δ 2.36 and δ 2.75 and a finely coupled doublet at δ 8.09. The former were a geminal methylene pair and the latter an olefinic proton "buto" a hetero-atom, similar to H5 in P5C. These signals, when carefully integrated and compared with the signal at δ 6.93, were present at equimolar equivalents to one another.

The diffusion spectra revealed that a significant set of signals was hidden under the water signal at δ 4.77. The sample was therefore heated to 310 K, causing the water signal to shift to a higher field and the multiplets thus became observable in the one-dimensional noesypresat spectrum. They integrated for a total of three protons when compared with the new doublet at δ 8.09.

At this point a series of heteronuclear experiments were measured, first on 31P to establish the presence of the phosphate group. Three clearly distinguishable triplets were observed, the largest at the lowest field δ 5.19 was attributed to the unreacted PP. The other two triplets at δ 5.02 and δ 5.08 were clearly new phosphate-containing molecules. Other signals were present but were overlapped by the major and minor components or at too low a concentration to be observed at the signal-to-noise level achieved.

The next step involved acquisition of an HMQC and HMBC 1H-13C correlated set of two-dimensional spectra (Fig. 5). The identity of the reaction products was deduced by combination of the two data sets. The coupled network of five carbons connected to the geminal methylene pair corroborated the earlier one-dimensional evidence and confirmed the olefinic functionality. The possibilities for the reaction products were thus narrowed down to one site of reaction on the P5C, namely the C-4 carbon. The other possibilities arising from either isomers of P5C or the ring opened form DL-glutamic-γ-semialdehyde (the latter would involve addition of the amine to the aldehyde of PP to form a Schiff base) were all excluded on the basis of the coupling network observed from the TOCSY and HMBC experiments.

The proposed new Structures 3a and 3b would have resulted from an addition of the C-4 carbon to the aldehyde of PP, reminiscent of the Knoevenagel reaction, with the imine function of P5C behaving like the carbonyl in the more usual ketone-aldehyde reaction. Following the condensation, elimination of water formed an olefin with two possible stereoisomers, one of which turned out to be the major product with a molecular mass of 342 Da. Distinction between these isomers was achieved using double-pulse field gradient noe (14) experiments as shown in Fig. 6B. Selective inversion...
of the resonance at \( \delta 6.93 \) resulted in a positive enhancement of the signals at \( \delta 8.09 \) and \( \delta 4.76 \), thus proving that the major product isomer had the configuration as shown in Structure 3a.

The other new components observed in the spectra were characterized in the same way. The precursor to Structures 3a and 3b was Structure 4 and was steadily converted to the olefins (Structure 3) on standing. Structure 4's molecular mass was 360 Da.

The spectrum in Fig. 3C was not definitive, because there are three chiral centers, and the eight diastereoisomers are paired into four NMR distinguishable structures. The spectrum obtained by HPLC-NMR established the presence of two NMR-distinguishable structures. The multiplets at \( \delta 4.74 \) and \( \delta 4.91 \) and their coupled partners from the one-dimensional TOCSY at \( \delta 2.11, -\delta 2.3 \) (obscured by methyls), and \( \delta 7.99 \) suggested the presence of two of the diastereomeric structures represented by Structure 4. We have found no evidence of the formation of the remaining two possible diastereoisomers, possibly because these are also transient products present at even

### Table I—continued

| Structure and accurate m/z ion observed | Assignment and Nucleus in bold | Chemical Shift (\( \delta \) ppm) |Multiplicity & Coupling Constants (J Hz or 2)|
|----------------------------------------|-------------------------------|----------------------------------|------------------------------------------|
| [\( \alpha \)] PP 248.142              |                               |                                  |                                          |
| 2                                     | C-CH\(_2\)                    | 147.8 q                         |                                          |
| 3                                     | C-CH\(_2\)                    | 182.2 q                         |                                          |
| 4                                     | C-CH\(_2\)                    | 199.2 q                         | 176                                      |
| 5                                     | C-CH\(_2\)                    | 129.0 m                         |                                          |
| 6                                     | CH                            | 127.8 d                         | 140                                      |
|                                         | CH                            | 7.81 s                          |                                          |

| [\( \omega \)] PP 248.142              |                               |                                  |                                          |
| 2                                     | C-CH\(_2\)                    | 147.8 q                         |                                          |
| 3                                     | C-CH\(_2\)                    | 185.4 q                         |                                          |
| 4                                     | C-CH\(_2\)                    | 136.5 q                         |                                          |
| 5                                     | C-CH\(_2\)                    | 91.7 q                          |                                          |
| 6                                     | CH                            | 128.9 d                         | 140                                      |
|                                         | CH                            | 7.77 s                          |                                          |

| [2] PSC 114.115                        |                               |                                  |                                          |
| 2                                     | C-CH\(_2\)                    | 81.5 d                          |                                          |
| 3                                     | C-CH\(_2\)                    | 35.5 q                          |                                          |
| 4                                     | C-CH\(_2\)                    | 41.6 t                          |                                          |
| 5                                     | CH                            | 177.6 d                         |                                          |
|                                         | CH                            | 7.78 t                          | 21.1, 12.2                               |
| 6                                     | CH-CHOCH                      | 187.3 s                         |                                          |

| [2] PSC 116.119                        |                               |                                  |                                          |
| 2                                     | C-CH\(_2\)                    | nd                              |                                          |
| 3                                     | C-CH\(_2\)                    | 45.1 d                          | 7.6, 6.5 and 2.5                        |
| 4                                     | C-CH\(_2\)                    | 17.7 dd                        | -12.9, 10.3, 7.6                        |
| 5                                     | CH                            | 7.76 t                          |                                          |
|                                         | CH                            | nd                              |                                          |
| 6                                     | CH-CHOCH                      | 187.3 s                         |                                          |

| [3c] Z isomer                          |                               |                                  |                                          |
| 2                                     | C-CH\(_2\)                    | 18.8 s                          |                                          |
| 3                                     | C-CH\(_2\)                    | 24.0 t                          |                                          |
| 5                                     | C-CH\(_2\)                    | 144.1 s                         |                                          |
| 6                                     | C-CH\(_2\)                    | 131.1 s                         |                                          |
| 10                                    | CH                            | 165.0 s                         |                                          |
|                                         | CH                            | 8.21 t                          | 2.2                                      |
| 11                                    | CH                            | 7.90 s                          |                                          |
|                                         | CH                            | 4.75 t                          | mo                                       |
|                                         | CH-CHOCH                      | 183 s                           |                                          |

| [3b] Z isomer                          |                               |                                  |                                          |
| 2                                     | C-CH\(_2\)                    | nd                              |                                          |
| 5                                     | C-CH\(_2\)                    | 64.4 d                          |                                          |
| 6                                     | CH                            | 124 s                           |                                          |
| 7                                     | CH                            | 6.66 s                          |                                          |
|                                         | CH                            | 123 d                           |                                          |
| 8                                     | CH                            | 150.0 s                         |                                          |
| 9                                     | CH                            | 171.7 s                         |                                          |
| 10                                    | CH                            | 8.21 t                          | 2.2                                      |
| 11                                    | CH                            | 7.90 s                          |                                          |
|                                         | CH                            | 4.75 t                          | mo                                       |
|                                         | CH-CHOCH                      | 183 s                           |                                          |

| Structure and accurate m/z ion observed | Assignment and Nucleus in bold | Chemical Shift (\( \delta \) ppm) |Multiplicity & Coupling Constants (J Hz or 2)|
|----------------------------------------|-------------------------------|----------------------------------|------------------------------------------|
| [\( \beta \)] 266.257                 |                               |                                  |                                          |
| 2                                     | C-CH\(_2\)                    | nd                              |                                          |
| 3                                     | C-CH\(_2\)                    | 18.9 d                          |                                          |
| 5                                     | C-CH\(_2\)                    | 2.46 s                          |                                          |
| 6                                     | CH                            | nd                              |                                          |
| 7                                     | CH                            | 129 s                           |                                          |
| 10                                    | CH                            | nd                              |                                          |
| 11                                    | CH                            | 8.0 s                           |                                          |
| 10a                                   | CH                            | nd                              |                                          |
|                                         | CH-CHOCH                      | 5.09 d                          |                                          |
|                                         | CH-CHOCH                      | 5.01 d                          |                                          |
|                                         | CH-CHOCH                      | 5.01 d                          |                                          |
|                                         | CH-CHOCH                      | 5.01 d                          |                                          |

| Structure and accurate m/z ion observed | Assignment and Nucleus in bold | Chemical Shift (\( \delta \) ppm) |Multiplicity & Coupling Constants (J Hz or 2)|
|----------------------------------------|-------------------------------|----------------------------------|------------------------------------------|
| [\( \gamma \)] 299.222                |                               |                                  |                                          |
| 2                                     | C-CH\(_2\)                    | nd                              |                                          |
| 3                                     | C-CH\(_2\)                    | nd                              |                                          |
| 4                                     | C-CH\(_2\)                    | nd                              |                                          |
| 5                                     | C-CH\(_2\)                    | nd                              |                                          |
| 6                                     | CH                            | nd                              |                                          |
| 7                                     | CH                            | 6.23 s                          |                                          |
| 8                                     | CH                            | nd                              |                                          |
| 9                                     | CH                            | nd                              |                                          |
| 10                                    | CH                            | 3.18 m                          |                                          |
| 11                                    | CH                            | nd                              |                                          |
|                                         | CH                            | 3.08 m                          |                                          |
lower concentrations than their isomers. From the NMR data obtained, we are unable to assign the stereochemistry of the observed diastereoisomers.

The third (minor) component consisted of the compounds with \( m/z \) of 297. Their identities were more difficult to prove, because they were present at low concentration. However, the HPLC-NMR-MS resolved them satisfactorily and confirmed their structures that of Structure 5.

The key information was the absence of any signal between 4 and 5 ppm and the presence of two mutually coupled pairs of methylene groups. Two isomeric forms of this compound were observed, the \( E \) and \( Z \) forms. However, owing to the small quantity (2 µg) present in the mixture, it was not feasible to prove which was the major isomer. The observed chemical shifts and coupling constants for the various components identified in the mixture at 310 K after 12 days of reaction are listed in Table I.

Concentrations of compounds in the reaction mixture after 36 h of incubation were PP 6.9 mM, P5C 4.6 mM, and the three products 1.14 mM (Structure 3a), 0.23 mM (Structure 3b), and 1.50 mM (Structure 4). To discover whether the same reactions occur at lower concentrations (closer to those expected \textit{in vivo}), aliquots of each stock solution were diluted 10-fold with \( D_2O \), mixed, and again incubated at 310 K. This reaction was monitored by \(^1\)H NMR and MS and confirmed that products were formed with similar chemical shifts in the \(^1\)H NMR and quasi-molecular ions of the same mass as those observed in the reaction at the higher concentration.

**Interaction of P5C with Other Aldehydes and Ketones**—We incubated P5C with a range of aldehydes and ketones and successfully formed condensation products with formaldehyde, acetaldehyde, propionaldehyde, butyraldehyde, valeraldehyde, benzaldehyde, pyruvic aldehyde, glyoxylic acid, pyruvic acid (methyl glyoxylic acid), acetoacetic acid, 2-oxobutyric acid, and oxaloacetic acid. New products were confirmed by gas chromatography-MS (GC-MS) of their trimethylsilyl (tms) derivatives. The first seven of these (all aldehydes) had products with the molecular mass predicted for di-tms derivatives. The second group of four acids had tri-tms, and the last, oxaloacetic acid, had a tetra-tms derivative of the corresponding P5C/carbonyl adduct (comparable with Structure 4 for P5C/PP). The key product was the acetoacetic acid/P5C adduct, molecular mass 432 Da, for the tri-tms derivative. We found the latter in the first urine sample collected from the child at the time of her acute illness. This specimen showed moderate ketonuria (4 mM) as medication, the alternative was that an endogenous compound was responsible. This was likely to be an intermediate that accumulates as a result of her primary inherited disorder. Biochemical conversions of amino acids by PP-dependent enzymes involve formation of a Schiff base between the amino acid and PP (5, 21). Because proline is an imino acid that does not react with the carbonyl group of PP (8, 21), it was unlikely that this was the de-activator, and we confirmed its non-reactivity by GC-MS and HPLC-MS. The alternative was P5C, which is in equilibrium with L-glutamic-\( \gamma \)-semialdehyde (Fig. 1). One possibility was formation of a Schiff base between the C-4 amino group of pyridoxamine and the carbonyl group of the semialdehyde. However, at physiological pH, the equilibrium would favor P5C, which would be the predominant form of the intermediate, and, in preliminary studies, we found no evidence of interaction between P5C and pyridoxamine. Pyridoxine and 4-pyridoxic acid were also inactive. Thus it was probable that, as for other reported vitamin B\(_6\) antagonists, the C-4 carbonyl group of PP was involved in the proposed interaction, and we confirmed this for PP \textit{in vitro} at physiological pH and temperature. Studies with pyridoxal were inconclusive, because its aldehyde group was de-activated by formation of a hemi-acetal (22) in our experimental conditions. However, this would not preclude the possibility of analogous reaction \textit{in vivo} for pyridoxal.

The P5C/PP conjugates that we synthesized and characterized have not been reported before. They are neither hydrazones nor oximes and represent a new class of vitamin B\(_6\) adducts. Covalent bonding with the PP aldehyde group would de-activate the vitamin. The mechanism of reaction has not been proven. However, based upon chemical knowledge of the system, we propose an addition of a proline-derived species to the aldehyde followed by a base-catalyzed elimination as shown in Scheme 1, to be the most likely sequence. We found definitive evidence for deuteration of P5C at C-4 in \( D_2O \), in that both methylene protons at C-4 of P5C disappeared in the 12-day mixture. In addition, the residual C-3 methylene protons show a greatly simplified apparent coupling pattern, as a result of the deuteron substitution for their vicinal partners (see the

**DISCUSSION**

Vitamin B\(_6\) deficiency was an unexpected, incidental, finding in this child, whose primary problem is the inherited disorder hyperprolinemia type II. The association has not been reported before. There was no question of nutritional deficiency, and the explanation, therefore, had to be antagonism or de-activation of the vitamin. A range of antagonists is known (5, 18, 19), and these have been categorized into three groups (19). First, structural analogues of vitamin B\(_6\) (for example, 4-deoxypyridoxine (20)); second, substances that are analogues of natural substrates for vitamin B\(_6\)-dependent enzymes and are converted to intermediates, which bind to the enzymes irreversibly (19); and third, carbonyl reactive reagents that react with the aldehyde group of PP and thereby block its coenzyme activity. These agents include hydrazines, which form hydrazones (examples are L-amino-D-proline in linseed oil and several drugs, including the anti-tuberculous agent isoniazid, carbidopa, phenelzine, and hydralazine), substituted hydroxylamines, which form oximes (for example, D-cycloserine and L-canaline), and sulphydryl compounds, including L-penicillamine (5, 18, 19).

Because the child was not receiving any vitamin antagonists as medication, the alternative was that an endogenous compound was responsible. This was likely to be an intermediate that accumulates as a result of her primary inherited disorder. Biochemical conversions of amino acids by PP-dependent enzymes involve formation of a Schiff base between the amino acid and PP (5, 21). Because proline is an imino acid that does not react with the carbonyl group of PP (8, 21), it was unlikely that this was the de-activator, and we confirmed its non-reactivity by GC-MS and HPLC-MS. The alternative was P5C, which is in equilibrium with L-glutamic-\( \gamma \)-semialdehyde (Fig. 1). One possibility was formation of a Schiff base between the C-4 amino group of pyridoxamine and the carbonyl group of the semialdehyde. However, at physiological pH, the equilibrium would favor P5C, which would be the predominant form of the intermediate, and, in preliminary studies, we found no evidence of interaction between P5C and pyridoxamine. Pyridoxine and 4-pyridoxic acid were also inactive. Thus it was probable that, as for other reported vitamin B\(_6\) antagonists, the C-4 carbonyl group of PP was involved in the proposed interaction, and we confirmed this for PP \textit{in vitro} at physiological pH and temperature. Studies with pyridoxal were inconclusive, because its aldehyde group was de-activated by formation of a hemi-acetal (22) in our experimental conditions. However, this would not preclude the possibility of analogous reaction \textit{in vivo} for pyridoxal.
Our preliminary observations that P5C also reacts with other aliphatic and aromatic aldehydes and ketones at physiological (i.e., slightly alkaline) pH suggest that this condensation may be a generic reaction of activated carbonyl compounds. Some of these interactions, for example P5C with acetoacetic acid, occur in vivo in hyperprolinemia type II.

At present, the evidence that the P5C/PP interaction demonstrated in vitro occurs in this child in vivo is circumstantial: namely, that she had vitamin B₆ deficiency without any apparent explanation other than life-long exposure to abnormally high concentrations of P5C. So far we have not been able to demonstrate any of the three new adducts in her plasma or urine in free form or as glucuronide, sulfate, or glycine conjugates by GC-MS (as tms derivatives) or by NMR. The adducts may be too unstable for extraction and derivatization for GC-MS analysis and the concentrations too low for NMR.

In vitro we demonstrated PP/P5C interaction at a P5C concentration of 0.46 mM. This is much higher than plasma P5C concentrations found normally (0.2–2.0 μM) and in hyperprolinemia type II (10–40 times normal) (8, 23, 24). P5C, however, is an intracellular metabolite produced in liver, kidney, and brain by the mitochondrial enzyme proline oxidase (no EC number assigned) (8). Normally it is then converted to glutamic acid by Δ¹-pyrroline-5-carboxylic acid dehydrogenase in the mitochondrial matrix (Fig. 1) (25–27). Some also diffuses into the cytoplasm, where it is reduced back to proline by P5C reductase (EC 1.5.1.2) generating NADP⁺ from NADPH and driving the pentose phosphate shunt (8, 28). Deficiency of P5C dehydrogenase in hyperprolinemia type II leads to a considerable increase in the cytoplasmic flux of P5C to proline, as evidenced by the very high proline concentrations observed (8) (2.29–2.96 mM). Because there is a large rapidly exchanging pool of vitamin B₆, the P5C/PP adducts form at pH 7.2, the usual cytosolic pH level (31). Consumption of PP in such a reaction would lead to evidence to the P5C dehydrogenase deficiency of P5C (see Fig. 1) may increase the risk of seizures in hyperprolinemia type II.

Our ongoing studies, however, indicate that P5C also forms adducts with other biologically important aldehydes and ketones. These include pyruvic acid, oxaloacetic acid, and acetoacetic acid, each of which are central intermediates in metabolism. We have shown P5C/acetoacetic acid adducts unequivocally in this child's urine and thereby demonstrated their production in vivo. With improved analytical sensitivity, we anticipate that other conjugates will be detected. An accumulation of P5C may, therefore, have previously unsuspected effects on body biochemistry. One or more of these adducts may also be implicated in seizures.

Our studies have demonstrated a novel interaction between P5C and PP, which would deactivate the vitamin. If this occurs in vivo, as we believe, P5C represents a unique endogenous vitamin B₆ antagonist. It remains to be seen whether other individuals with hyperprolinemia type II are also vitamin B₆ deficient. We advise that affected individuals have plasma PP and 4-pyridoxic acid measured, because correction of a deficiency may help to prevent seizures. It would also be important to search for other biologically important P5C conjugates in their blood and urine.

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Pyridoxal Phosphate De-activation by Pyrroline-5-carboxylic Acid: INCREASED RISK OF VITAMIN B6 DEFICIENCY AND SEIZURES IN HYPERPROLINEMIA TYPE II

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