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Nickel Complexes of Sepiapterin and 6-Acetyldihydrohomopterin, a Pyrimidodiazepine from Drosophila[1,2]

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The nonfluorescent pyrimidodiazepine in *Drosophila melanogaster* 6-acetyldihydrohomopterin (H_2Ahp) was studied using ultraviolet and infrared spectroscopy. The H_2Ahp was unstable in 3% NH_4Cl whereas a related pteridine sepiapterin was stable. Since Ni^{2+} stabilized H_2Ahp completely, the structure of the $H_2Ahp \cdot Ni$ complex was examined. Among 15 pterins, including sepiapterin, the spectral properties in the presence of Ni^{2+} reflect the pK_a's and the reactive group on the side chain but for H_2Ahp the spectral properties are rather different from the pteridines and they indicate that the seven-membered ring seemed to have the predominant influence. The Ni^{2+} comples of H_2Ahp resulted in a shift in the absorption maximum from 383 to 436 nm. The corresponding spectral shift of the pteridines due to Ni^{2+} was much less. From the infrared spectra of H_2Ahp and sepiapterin in the presence and absence of Ni^{2+}, the sites of interaction of Ni^{2+} with H_2Ahp were shown to be the phenolic oxygen and N5 in the ring. In the absence of Ni^{2+} an internal hydrogen bond in sepiapterin was indicated that may involve the carbonyl oxygen and the secondary alcoholic oxygen on the side chain. Other metal ions were tested (Cd^{2+}, Zn^{2+}) but were not as effective as Ni^{2+} in stabilizing H_2Ahp.

INTRODUCTION

The most common sources of diazepines in nature are bacteria and fungi (*1*). In *Drosophila melanogaster*, two diazepines occur. One is the eye pigment, drosopterin, a complex heterocyclic molecule, a part of which is the seven-membered diazepine ring (*2*). The second is 6-acetyldihydrohomopterin (H_2Ahp)[4] a pyrimidodiazepine that is the metabolic precursor to drosopterin (*3, 4*). The biosynthesis of this intermediate involves the conversion of GTP to dihydroneopterin triphosphate and of the latter to 6-pyrovoyl-tetrahydropterin (*5–7*). The latter compound

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[4] Abbreviations used: H_2Ahp, 6-acetyldihydrohomopterin; Pipes, 1,4-piperazinediethanesulfonic acid.

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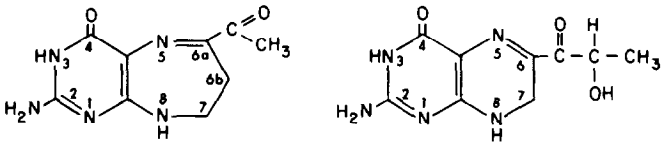

FIG. 1. Structural formulas.

is the immediate metabolic precursor of H_2Ahp and, perhaps less directly, of sepiapterin (*4–8*). The structure of H_2Ahp was established (*3, 9*) and has certain features that are in common with the structure of sepiapterin (Fig. 1). The chemistry of the pyrimidodiazepine structure in H_2Ahp is of interest. This report describes the instability of H_2Ahp in certain conditions and the stabilizing effect of divalent cations. The comparable properties of related pterins are also reported. Models for the metal complexes are proposed.

EXPERIMENTAL PROCEDURES

Chemicals. We obtained KBr (spectral grade) from Harshaw, Pipes from Research Organics, Inc., and $NiCl_2$ from Baker. To prepare a 1 M solution, $NiCl_2$ was dissolved in 0.07 M HCl and then the pH was adjusted to 3 with NaOH. More dilute solutions of $NiCl_2$ are stable at higher pH levels.

The isolation of H_2Ahp and sepiapterin from Drosophila heads was described earlier (*9*). Deoxysepiapterin (isosepiapterin) was obtained from two sources: one as a byproduct of the H_2Ahp isolation, and, second, together with propionylpterin as a generous gift from Dr. W. Pfleiderer. Neopterin 3′-monophosphate was a generous gift from Dr. T. Shiota. Formylpterin was obtained by treatment of biopterin with $NaIO_4$. Oxidized sepiapterin (6-lactoyl pterin) was obtained after the spontaneous oxidation of sepiapterin in water exposed to air; its identity was confirmed by the uv spectra (*10*) after separation from sepiapterin on a cellulose microgranular column (20 × 0.7 cm) with water. Dihydroneopterin 3′-monophosphate and dihydroformylpterin were obtained by reduction of the respective oxidized pterins with Zn dust in alkali (*11*) and removing the oxidized Zn^{2+} with a Chelex column (3 × 1.7 cm) by elution with 1 mM NaOH. Dihydroformylpterin was further purified by chromatography on Sephadex G-25 column (25 × 1.7 cm i.d.) equilibrated and eluted with water. Yellow and blue fluorescent pteridines were separated (*12, 13*). The yellow fractions were pooled and the uv spectrum was as expected for 7,8-dihydro-6-(1′-carbonyl) pterin (*14*). All other pterins were obtained from Sigma. Solutions were routinely protected from light.

Chromatography. The C18 μBondapak column (Waters Assoc.) was used in conjunction with a Waters 6000A pump and U6K injector. The absorbance at 260 nm and fluoresence (excited at 360, >418 nm for emission) were maintained with Schoeffel 770 and 970 instruments as described earlier (*15*).

Infrared spectra. Infrared spectra were obtained by the diffuse reflectance technique (1) using a Digilab FTS-20/C (Digilab, Division of Bio-Rad Laboratories,

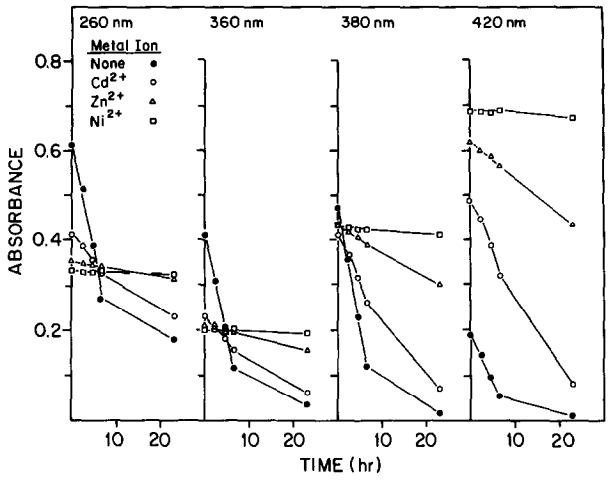

FIG. 2. Stabilization of H_2Ahp in NH_4Cl by Divalent Metal Ions. H_2Ahp (0.038 mM) was dissolved in 3% NH_4Cl that contained 10 mM concentration of Cd^{2+}, Zn^{2+}, or Ni^{2+}, as shown. The solution was incubated in the dark at 25°C for the times shown.

Cambridge, Mass.) FTIR spectrometer equipped with a high-intensity Globar source, a triglycine sulfate detector, a Model 496 Michelson interferometer with a KBr beam splitter, and the associated data system. The diffuse reflectance attachment was obtained from Digilab and was a JASCO Model DRA-100.

Samples were prepared by lyophilizing solutions of the compound of interest that also contained $NiCl_2$ at various concentrations along with 100 mM KBr and transferring this mixture directly to the diffuse reflectance sample cup with no further treatment. The spectra result from the accumulation of 1000 scans obtained at a resolution of 1 cm^{-1} with the sensitivity set to the highest value consistent with avoiding an overload of the analog-to-digital converter. Upon completion of the infrared study, the H_2Ahp and sepiapterin were recovered from the salt. Their uv spectral and chromatographic properties were unchanged.

RESULTS

Instability of H_2Ahp in 3% NH_4Cl. The chromatographic resolution of H_2Ahp and sepiapterin was the final problem in purification of H_2Ahp (*9*). Since the cellulose TLC, developed with 3% NH_4Cl, resolved these two compounds in 45 min a cellulose column was employed with the same developer. However, the recovery of H_2Ahp after 15–20 h was poor, although that of sepiapterin was excellent. Therefore the stability of H_2Ahp in 3% NH_4Cl (pH 4.9) was studied by observing its absorbance at four wavelengths (Fig. 2); at the several wavelengths the deterioration of H_2Ahp could be observed. In preliminary studies the pH was varied from 4.9 to 7.5. The H_2Ahp degraded more slowly as the pH increased (one-half as fast as pH 7.5) but no condition was found that gave good stability.

Also, the chromatographic resolution decreased as the pH increased. The addition of certain divalent metal ions did provide stabilization at pH 4.9. As shown in Fig. 2 the divalent cations Cd^{2+}, Zn^{2+}, and Ni^{2+} all stabilized H_2Ahp in 3% NH_4Cl; Ni^{2+} provided excellent results for 23 h. From the marked decrease in absorbance at 260 nm and the increase at 420 nm at zero time it would appear that the metal ion complexes immediately with H_2Ahp; the greater a given metal ion caused a change in absorbance the more stabilization was obtained. Whether Cd^{2+} and Zn^{2+} had lower dissociation constants or were less effective than Ni^{2+} was not investigated. The characteristics of the Ni^{2+} complex with H_2Ahp are described in the following sections after briefly comparing chromatographic behavior and the fluorescent and phosphorescent properties of H_2Ahp with a number of pterines.

Chromatographic properties. The resolution of several pterins by reversed-phase liquid chromatography was accomplished by 10% CH_3OH using a C18 column. Using conditions similar to Fukushima and Nixon (*16*), the following pterins eluted in the order listed, exhibited symmetrical peaks, and were completely resolved from one another: pterin-6-carboxylic acid, xanthopterin, 6-hydroxymethylpterin, 6-formylpterin, pterin, 6-methylpterin, lactoylpterin, sepiapterin, deoxysepiapterin, propionylpterin. The chromatographic behavior of H_2Ahp under these conditions is quite different is that it is strongly retained by the column and, upon elution, the absorbance peak is skewed and poorly defined. This illustrates the relative polarities of the pterins and H_2Ahp as well as the anomolous behavior of the latter.

Fluorescence and phosphorescence. Earlier studies using cellulose thin-layer chromatography showed that H_2Ahp (called "quench spot" in those studies) had a R_f similar to sepiapterin in several solvents (*17*). A marked difference between H_2Ahp and the accompanying pterins from Drosophila eyes was the failure of the former to exhibit fluorescence under conditions in which the pterins did. After chromatography with certain solvents that contained acidic alcohol, a bright green fluorescence of the H_2Ahp did occur on the dried chromatogram. In the case of most solvents where fluorescence did not occur, a strong yellowish-green fluorescence was obtained by immersing the dried chromatogram in liquid nitrogen. The fluorescence of sepiapterin was strong and yellow at 25 and −196°C. If the chromatogram was developed in the presence of air, the sepiapterin was partially oxidized (and migrated somewhat faster), and this form has a blue-white fluorescence at −196°C. Sepiapterin and oxidized sepiapterin were separable in several solvents.

Phosphorescence was observed following chromatography on a cellulose thin-layer sheet using 3% NH_4Cl; the dried sheet was immersed in liquid nitrogen and illuminated with 360-nm light. Upon extinguishing the illumination, phosphorescent compounds emitted light for a few seconds. H_2Ahp was not phosphorescent at −196°C and neither was sepiapterin. A number of pterins were examined in this manner. Phosphorescence was observed for oxidized sepiapterin, pterin, 6-methylpterin, 6-hydroxymethylpterin, pterin-6-carboxylic acid, 6-formylpterin, biopterin, neopterin, neopterin monophosphate, and 6-propionylpterin. No phosphorescence was observed for H_2Ahp, sepiapterin, isoxanthopterin, xanthopterin, leucopterin, 7,8-dihydroformylpterin, neodrosopterin, drosopterin, isodroso-

pterin, and aurodrosopterin. When the chromatography sheet was at 25°C, no phosphorescence of any of the compounds occurred.

Oxidation and reduction. Presuming that oxidation of H_2Ahp would occur on the ring, MnO_2 was added to a solution of this compound in 10 mM NaOH. No new spectral features arose; indeed, absorption at all wavelengths from 260–300 nm and 330–420 nm decreased steadily for 100 h; the absorption at 310 nm remained constant.

Reduction of H_2Ahp in 10 mM NaOH by $NaBH_4$ (10 mM) occurred rapidly (9), resulting in a loss of absorption bands from 340–440 nm and a decrease and shift of the 265 nm peak to 283 nm. Reduction of the ketone side chain and the diazepine ring structure are both possible and these spectral changes cannot distinguish between the two possibilities. Addition of $NaBH_4$ to the product of oxidation by MnO_2 did not restore any absorption, but merely further diminished the absorption remaining at 340–420 nm. The reduction of typical pterins to the tetrahydro form is accompanied by a bleaching in the 320 to 400-nm region of the spectrum.

Extinction coefficients. A stock solution of H_2Ahp was prepared in 10 mM phosphate (pH 7.0) at 50°C that had an absorbance of 6.6 at 383 nm. A linear relationship between absorbance and concentration was observed for absorbances at 383 nm of 1.0 or less; at higher concentrations the absorbance was less than expected. The extinction coefficients (mM^{-1} cm^{-1}) in 10 mM phosphate, pH 7.0, were 16.5 at 260 nm and 12.8 at 383 nm; in methanol they were 16.0 at 259 nm and 11.9 at 386 nm. These are based on absorbances in the linear range.

Effect of $NiCl_2$ concentration. Solutions of H_2Ahp in methanol or water (1.0 ml) were placed in cuvettes and aliquots (5 μl) of $NiCl_2$ (1.9 M, pH 3) were added incrementally. The spectra were obtained before and after the last addition of $NiCl_2$ (Fig. 3a, b) and the progressive decrease at 360 and increase at 410 nm with each addition of $NiCl_2$ are shown in Fig. 3c. Since the volume changes due to addition of $NiCl_2$ were small, no correction for dilution was made. The pH did not vary from 5.5 ± 0.5. The spectrum of neither H_2Ahp nor Ni · H_2Ahp changes significantly between pH 4 and 7.5. With H_2Ahp dissolved in methanol, $NiCl_2$ caused the absorbance at 360 nm to decrease more abruptly than the 410-nm absorbance increased (Fig. 2c). In water the 383-nm maximum was accompanied by a secondary maximum at 360 nm whereas in methanol the 360- and 383-nm maxima were nearly equal. Upon the complete formation of the Ni^{2+} complex a symmetrical peak with a 425-nm maximum replaced the more complex absorption curves seen in the absence of Ni^{2+} in either solvent. The Ni^{2+} complex also had a decreased absorption at 265 nm and a stronger peak at 315 nm. The formation of the Ni^{2+} complex was immediate in methanol but was noticeably slower in water; spectral readings were taken after stable values were observed.

The ability of a variety of pterins to form a complex with Ni^{2+} was measured by changes in the electronic spectra. The normal electronic spectra of many pterins have one maximum in the region of 250–290 nm and another at 320–420 nm. The spectral changes for biopterin and xanthopterin are shown in Fig. 4 as a function of the addition of Ni^{2+} or of shifting the pH from 7 to 13.

Comparison of nickel complexes of H_2Ahp and pterins. The spectral characteristics of H_2Ahp and a number of pterins in the presence and absence of Ni^{2+} and at

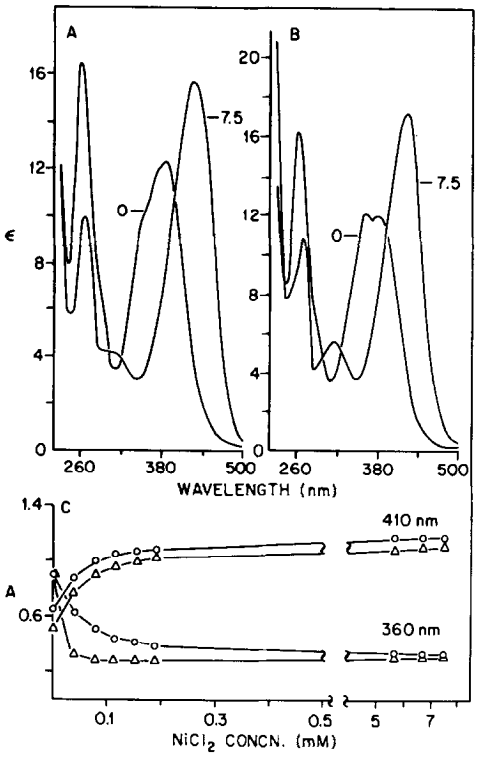

FIG. 3. Spectra of 6-acetyldihydrohomopterin. The sample was dissolved in water or methanol in a total volume of 1.0 ml to which was added 5-μl aliquots of 1.9 M $NiCl_2$. After eight such additions the concentration of $NiCl_2$ was 7.5 mM. The uv-absorption spectra in 0 and 7.5 mM $NiCl_2$ are shown for water (A) and methanol (B). The absorbance, uncorrected for dilution, at 360 and 410 nm for both solutions resulting from each addition of $NiCl_2$ is shown in (C). Water solution, (○); methanol, (△).

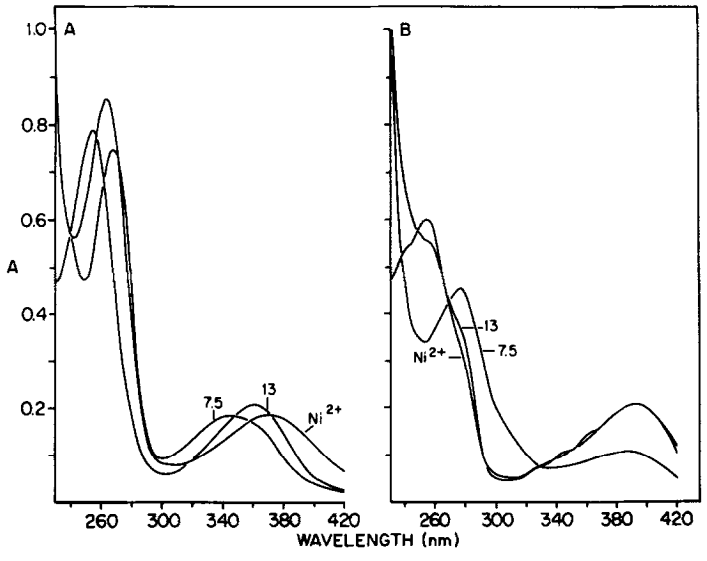

FIG. 4. Spectra of biopterin (A) and xanthopterin (B). The samples were dissolved in 50 mM Pipes (pH 7.5), 100 mM NaOH (pH 13), or 50 mM Pipes/5 mM $NiCl_2$ (pH 7.5).

TABLE 1

CHANGES IN SPECTRAL CHARACTERISTICS OF SEVERAL PTERINS AND 6-ACETYLDIHYDROHOMOPTERIN CAUSED BY Ni^{2+} OR ALKALINE pH

Substance (substituent at 6 position)	Absorption maximum at pH 7.5			Ratio of extinction coefficients at absorption maxima (+Ni/−Ni)	Absorption maximum at pH 12 (nm)
	$-Ni^{2+}$ (nm)	$+Ni^{2+}$ (nm)	Δ (nm)		
1. Pterin (H)	343	370	27	1.70	360
2. 6-Methylpterin (CH_3)	347	373	26	1.13	365
3. 6-Hydroxymethylpterin (CH_2OH)	347	372	25	1.05	363
4. Pterin-6-carboxylic acid (COOH)	345	372	27	1.04	365
5. 6-Formylpterin (CHO)	365	372	7	0.99	367
6. Biopterin ($CHOHCHOHCH_3$)	343	370	27	0.98	363
7. Neopterin monophosphate ($CHOHCHOHCH_2OPO_3^{2-}$)	345	373	28	1.09	—
8. Isoxanthopterin (7-oxypterin)	333	340[c]	7	1.78	339
9. Xanthopterin (6-oxypterin)	387	392	5	1.65	392
10. Leucopterin[a]	336	341[d]	5	2.04	341[e]
11. 7,8-H_2Neopterin monophosphate[b]	328	337	9	0.97	(329)[f]
12. Sepiapterin[b] ($COCHOHCH_3$)	419	426	7	0.93	438
13. Deoxysepiapterin[b] ($COCH_2CH_3$)	408	410	2	1.005	430
14. 7,8-H_2-6-Formylpterin[b]	420	420	0	1.00	—
15. 6-Propionylpterin ($COCH_2CH_3$)	360	360	0	1.00	—
16. 6-Acetyldihydrohomopterin ($COCH_3$)	383	436	53	1.63	400

Note. The substances were each dissolved in 50 mM Pipes (pH 7.5) to give an absorbance of 0.3–1.0 at the absorbance maxima. After the spectrum was recorded, 25 μl of 1 M $NiCl_2$ was added to 5 ml to give 5 mM Ni^{2+}. For the pH 12 value the substance was dissolved in 10 mM NaOH. The spectra were recorded on a Beckman DU-8 spectrophotometer.

[a] 2,4,6-oxypterinone.

[b] These all are reduced at the 7,8 position of pterin.

[c] Shoulder at 352 nm.

[d] Two minor peaks at 328 and 358 nm, respectively.

[e] Maximum for the dianion species.

[f] This value has been found for 7,8-H_2biopterin, and it is presumably the same for 7,8-H_2neopterin.

two pH values are summarized in Table 1. Two main features are reported: (1) the shift of the absorption maximum wavelength caused by Ni^{2+} and (2) the ratio of the extinction coefficients in the presence and absence of Ni^{2+}. Ni^{2+} complexes of pterin and of the 6-alkylpterins with oxidized rings (Nos. 1–7) give a maximum

around 372 nm, an ~27-nm increase from the maximum observed without Ni^{2+}. An exception is 6-formylpterin for which the increase in the maximum is much smaller, although the final position is still 372 nm; in the absence of Ni^{2+} at pH 7.5 the spectrum of 6-formylpterin has a major contribution from the anionic form, which absorbs maximally at longer wavelengths. Pterin differs from the others of this group (Nos. 1–7), in that a larger increase (1.7×) in the extinction coefficient occurs as a result of formation of the Ni^{2+} complex.

The three oxypterins (Nos. 8–10) gave a slight change in the wavelength for the absorption maximum, but gave the largest increase in extinction coefficient of all pterins examined due to Ni^{2+}. Of special note for this group is the virtual identity of the wavelength for the absorption maximua at pH 12 as compared to that of the Ni^{2+} complex.

Among the dihydropterins (Nos. 11–14) only 7,8-dihydroneopterin monophosphate seems to bind Ni^{2+} strongly since it is the only one of this group for which the absorption maximum of the Ni^{2+} complex was not less than the maximum at pH 12. The increment caused by Ni^{2+} in the absorption maximum wavelength for sepiapterin, deoxysepiapterin, and 7,8-dihydro-6-formylpterin decreased progressively in this subgroup with the last member showing no increase at all, indicating a failure to form a Ni^{2+} complex under these conditions.

Of particular interest are the changes that H_2Ahp (No. 16) undergoes; it had the largest Ni^{2+} induced bathochromic shift, 53 nm, and was unique among these compounds in that the maximum at pH 12 was 36 nm less than that for the Ni^{2+} complex. The increase in the extinction coefficient caused by Ni^{2+} (1.63×) is similar to that of pterin and the oxypterins. These data were obtained at pH 7.5 and thus differ slightly from spectral properties seen in Fig. 3.

The association constants of H_2Ahp and sepiapterin for Ni^{2+} were determined in 90 mM Pipes, pH 6.7. Titration of H_2Ahp (0.08 mM) with $NiCl_2$ was monitored at 420 nm and an association constant of 3×10^4 M^{-1} was calculated. Titration of sepiapterin (0.22 mM) with $NiCl_2$ was monitored at 480 nm, the region of maximum spectral shift, and an association constant of 1.4×10^2 M^{-1} was obtained. In the case of H_2Ahp and the Ni · H_2Ahp complex there was no appreciable change in spectrum between pH 4 and 7.5; below pH 4 H_2Ahp was unstable as shown by the steady decrease in absorbance at 380 nm. In the case of sepiapterin and the Ni–sepiapterin complex there were marked spectral shifts with pH. For the Ni–sepiapterin complex a p*K* occurs above pH 7.4 but its exact value was difficult to measure because nickel precipitated at pH values above 8. These data show that association constants of Ni^{2+} for H_2Ahp and sepiapterin differ by more than 4000-fold at pH 6.7 and that the latter exhibits proton dissociation above neutral pH.

Solutions of H_2Ahp were prepared in 0.1 M KBr and various concentrations of $NiCl_2$; the electronic spectra, shown in Fig. 5A, show the progressive formation of the nickel complex. No further changes occurred with larger concentrations of $NiCl_2$ (see Fig. 3). These solutions were lyophilized and transferred to the diffuse reflectance sample cup of the FTIR spectrophotometer. In Fig. 6 the mid-infrared spectrum of H_2Ahp is compared to the spectrum of H_2Ahp equilibrated with the largest concentration of Ni^{2+}. The difference spectrum resulting from subtracting the H_2Ahp spectrum from the Ni · H_2Ahp spectrum indicates a significant interac-

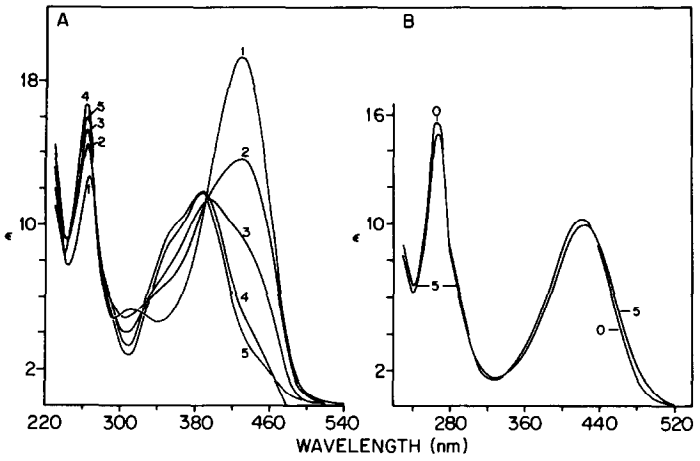

FIG. 5. Spectra of 6-acetyldihydrohomopterin and sepiapterin. (A) H_2Ahp (26 μM), 100 mM KBr, and various concentrations of $NiCl_2$ (1, 0.5 mM; 2, 0.1 mM; 3, 0.06 mM; 4, 0.01 mM; 5 none). Light path was 5 mm. (B) Sepiapterin (19 μM) in 100 mM KBr and 0 or 5 mM $NiCl_2$. Light path was 10 mm.

tion between Ni^{2+} and H_2Ahp, as evidenced by the shifting of bands, changes in band intensity, and the appearance or disappearance of bands. These changes, which occur primarily between 700 and 1700 cm^{-1}, are shown with an amplified scale in Fig. 7. The effects of intermediate concentrations of Ni^{2+} were also measured (data not shown), and we observed a progressive change in those bands as the Ni^{2+} concentration increased. Perhaps lyophilization avoids conditions for allowing the H_2Ahp to react with the Ni as the water is removed.

In contrast to the H_2Ahp, Ni^{2+} had practically no effect on the vibrational bands of sepiapterin as illustrated in Fig. 8. In fact, the only significant bands that appear

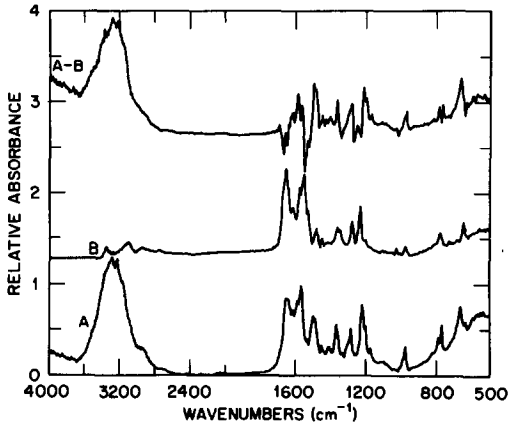

FIG. 6. Infrared spectra of H_2Ahp equilibrated with Ni^{2+} (A), H_2Ahp without Ni^{2+} (B), and the difference spectrum (A–B). Solutions examined in Fig. 5A were lyophilized and used for infrared studies. The spectrum in (A) was not corrected for the spectrum of $Ni(H_2O)_6^{2+}$, which made a very minor contribution compared to the other changes.

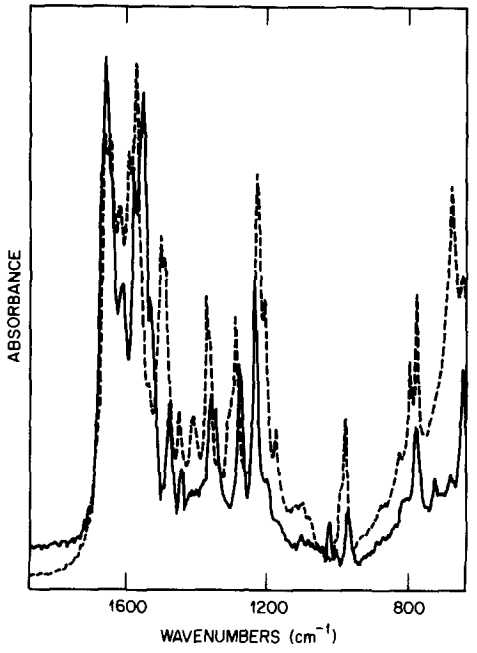

FIG. 7. Direct comparison of the infrared spectra of H_2Ahp before (----) and after (—) equilibration with Ni^{2+} (enlargement of plots in Fig. 6). Spectra have been normalized in order to approximate quantitative differences in band intensities.

in the difference spectrum are the bands one might expect from the hexaquo complex of Ni^{2+}, namely bands in the region of 3500, 1600, and 650 cm^{-1}. The location of the main band structures in the spectra shown in Figs. 6–8 are listed in Table 2. Similarly the electronic spectrum of sepiapterin was not affected appre-

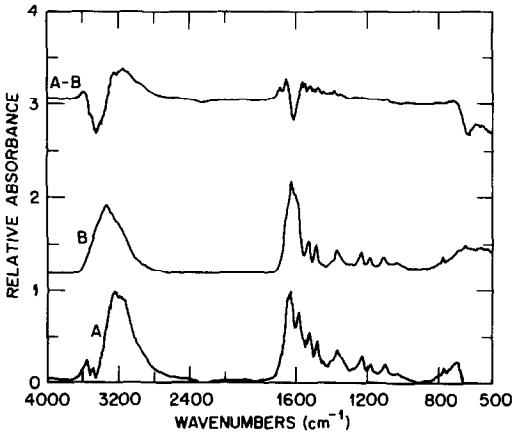

FIG. 8. Infrared spectra of sepiapterin equilibrated with Ni^{2+} after the spectrum of $Ni(H_2O)_6^{2+}$ has been subtracted (A); sepiapterin (B); and the difference spectrum (A − B). Solutions examined in Fig. 5B were lyophilized and used for infrared studies.

TABLE 2

LOCATION OF PRINCIPAL BANDS[a] IN THE INFRARED SPECTRA

6-Acetyldihydrohomopterin		Sepiapterin	
Without Ni^{2+}	With Ni^{2+}	Without Ni^{2+}	With Ni^{2+}
—	1663	—	—
1658	1653	—	—
—	1647	1630	1643
1619	1623	—	—
1581	1595	—	1590
1561	1575	—	—
1556	—	—	1548
1536	—	1531	—
—	1505	—	—
—	1495	—	—
1479	—	1489	1487
1446	1451	—	1450
—	1410	—	—
1360	1373	1372	1376
1348	—	—	—
1279	1289	—	—
1234	1225	1233	1233
—	1206	—	—
—	1175	1185	1184
—	—	1104	1101
1026	—	—	—
976	979	—	—
—	795	—	—
780	775	776	775
—	673	—	—
648	642	—	—

[a] Band locations are expressed as wavenumber (cm^{-1}). A group of peak locations enclosed by a bracket indicates a band structure with two or more slightly resolved, but prominent, maxima. The values were obtained by expanding the scales of the spectra so that accurate measurements of wavenumbers could be made (Fig. 7).

ciably by a Ni^{2+} concentration that caused maximal spectral change for H_2Ahp (Fig. 5B).

DISCUSSION

The instability of 6-acetyldihydrohomopterin in 3% NH_4Cl is in marked contrast to the stability of sepiapterin. Stabilization with divalent metal ions was employed since metal complexes with purine nucleotides are quite stable (*18*). Also diazepines are used to stabilize square planar and square pyrimidal geometries of certain divalent metal ions (*19*). The spectral properties of H_2Ahp demonstrate the inter-

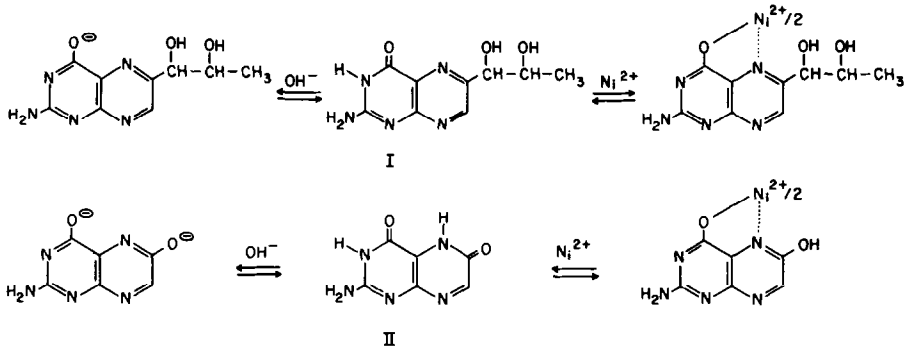

FIG. 9. Molecular species postulated for the Ni^{2+} complexes. Structures postulated for biopterin (I) or xanthopterin (II) at pH 7.5, pH 12, or as the Ni^{2+} complex at pH 7.5. The complex consists of dimeric pterins and one Ni^{2+}.

action with Ni^{2+} and, to a lesser extent, with Zn^{2+} and Cd^{2+}. The square planar configuration of Ni^{2+} is common but could not be confirmed here since attempts to crystallize the $Ni^{2+} \cdot H_2Ahp$ complex, or H_2Ahp alone, were unsuccessful. The $Ni^{2+} \cdot H_2Ahp$ complex was readily disrupted by EDTA and the pyrimidodiazapine recovered (*9*).

Assessment of the ability of a number of pterins to form a nickel complex was made to gain insight into the H_2Ahp complex. Hemmerich (*20*) showed that lumazines (2,4,6-oxypterinones) chelated Ni^{2+} such that the complex contained one Ni^{2+}, two lumazine, and two water molecules. The Ni^{2+} is bound covalently to the oxygen on C_4 and by charge transfer to N_5. We shall assume a similar stoichiometry for the pterins and evaluate H_2Ahp for sites of interaction with Ni^{2+}.

When pterins complexed with Ni^{2+} the absorption maximum shifted to a wavelength close to the maximum found at pH 12 (Table 1). Apparently the bound Ni^{2+} forms a complex with the oxygen on C_4 and the resulting electron configuration at N_3 resembles that formed at pH 12. These structural relationships are illustrated in Fig. 9.

The pterins examined did not respond uniformly to Ni^{2+} as indicated by the changes in the absorption maximum and the extinction coefficient at the maxima. One class is composed of pterin, isoxanthopterin, xanthopterin, and leucopterin; their absorbance, due to the Ni^{2+} complex, increased more than 1.6-fold. These pterins have no carbon side chain on C_6; pterin and isoxanthopterin have a hydrogen, whereas xanthopterin and leucopterin have an oxygen on C_6. The remaining pterins in Table 1 have a carbon substituent on C_6 which would appear to sterically hinder the binding of Ni^{2+} since the absorbance change due to Ni^{2+} was slight (0.93–1.13).

To explain the difference in affinity for Ni^{2+} among the pterins, we shall consider the three factors used by Hemmerich (*20*): (a) tautomerization between N_3 and the oxygen on C_4 and dissociation of a proton from that oxygen, (b) the basicity of N_5, and (c) the size of the substituent on C_6. Protonation of pterins usually occurs on N_1, not N_5, but the pK_a of this protonation will be used as an

indirect measure of the basicity of the rings and so of N_5. The pK_a values are from Pfleiderer (*14*).

Under the conditions used in Table 1 there are three pterins that complex Ni^{2+} only weakly as evidenced by a change ≤ 7 nm for the absorption maximum and a change in absorbance that is negligible (0.93–1.0). These three all have a keto carbon attached to C_6 and are 7,8-dihydropterins. Sepiapterin and deoxysepiapterin have a pK_{a_N} for the proton on N_1 of 1.3 and a pK_{a_O} for the phenolic group on C_4 of 10.0. We assume that 7,8-H_2-6-formylpterin has similar values. By way of contrast the group of oxidized pterins, e.g., biopterin and pterin, have a pK_{a_N} of 2.2 and pK_{a_O} of 7.9. Thus when N_1 (and N_5) are more acidic and/or the phenolic group is more basic, the Ni^{2+} complex is weak or nonexistent.

In the group consisting of xanthopterin, isoxanthopterin, and leucopterin the shift in the absorption maximum was small (5–7 nm) but the increase in absorbance large (1.65–2.04). For this group the pK_{a_N} values are low (1.6, −0.5, and −1.66, respectively) and the pK_{a_O} is 9.5. However, these three compounds have an intermediate pK_a around 7 due to the dissociation of the phenolic group on C_6 of the pyrazine ring. From the comparison of the pK_a values of these three pterins to those of sepiapterin, it would seem that the important factors for the formation of the Ni^{2+} complexes are not only the acidity of the OH on C_4 and the basicity of the N_5, but the amophoteric properties of the active molecule as well. The position of the charge is not fixed because the conjugated system of double bonds can supply electrons to the oxygen on C_4 and to N_5 from the atoms that possess them in excess.

The Ni^{2+} complex of H_2Ahp exhibited the largest shift in the absorption maximum (53 nm) of all compounds examined and also the largest increase in the extinction coefficient. Also the maximum at pH 12 was 36 nm lower than that obtained for the Ni^{2+} complex, an additional unique feature among the 16 compounds of Table 1. In comparison with sepiapterin the Ni^{2+} complex of H_2Ahp has a higher absorption maximum (436 vs 426 nm) and a larger increase in absorbance (53 vs 7). The structure of H_2Ahp is similar to that of sepiapterin and the bathochromic shift in the absorption maximum from pH 7.5 to 12 for these two compounds is similar (17 and 19 nm, respectively). However, there is a marked difference between the two compounds in that the absorption maximum at pH 7.5 and 12 is 400 nm or less for H_2Ahp as compared to 419 nm or greater for sepiapterin. This difference in the alkaline absorption maximum for H_2Ahp and sepiapterin may be the result of the disortion and strain on the chromophore caused by the existence of the seven-membered ring in the former. When H_2Ahp formed a chelate with Ni^{2+} the absorption maximum shifted to 436 nm, 36 nm higher than in alkali, and very close to the maximum for sepiapterin in alkali. We suggest that the formation of the Ni^{2+} complex by H_2Ahp alters the electronic structure in the diazapine ring, and thus the absorption maximum for its chromophore. The strong complex with Ni^{2+} may be facilitated by the geometry of the diazapine ring as well as the proton dissociation constants of H_2Ahp, pK_{a_N} 2.7, and pK_{a_O} 10.7 (*9*). The strain existing in the diazepine ring may be the cause of the instability of H_2Ahp when it is dissolved in 3% NH_4Cl; the relief of strain in the Ni^{2+} complex may then be associated with electronic rearrangement of the pyrimidodiazepine. Since the

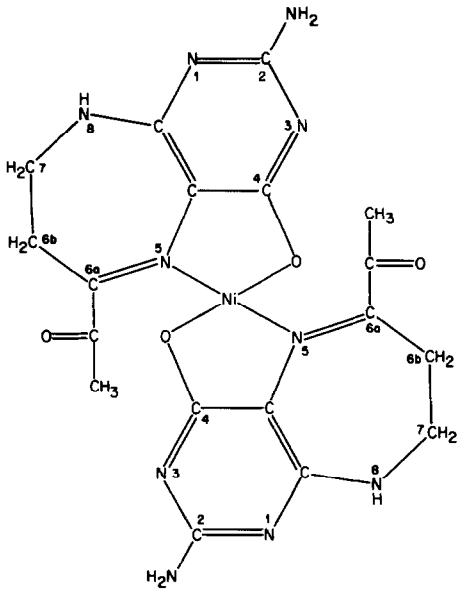

FIG. 10. A Ni^{2+} chelate formed between two molecules of H_2Ahp and one Ni^{2+} ion.

dissociation of the Ni^{2+} complex by EDTA occurs readily and H_2Ahp is recovered, there can be no covalent structural alterations produced in the complex. The degradation product(s) that result from action of weak acid or NH_4Cl on H_2Ahp will be of interest to determine the route of the such reactions.

The location of the H_2Ahp-binding sites for Ni^{2+} was examined by infrared. A chelate with the structure shown in Fig. 10 is considered likely. Such a structure would be consistent with the vibrational spectra shown in Fig. 6 where a new band structure appears at 1495 and 1505 cm^{-1} in the Ni · H_2Ahp spectrum and appears as a prominent peak in the difference spectrum. These bands are not present in the H_2Ahp spectrum (see Table 2). The energy of this band (1500 cm^{-1}) is too low for pure double bonds (C=O, C=C) but would be consistent with the hybrid bonds C····O——Ni which could exist in the structure shown in Fig. 10. In fact theoretical band assignments obtained from calculations made for metal chelates with acetylacetone have assigned the C····O in the chelate ring to bands ranging from 1450 to 1598 cm^{-1} (*21*). Thus the appearance of the new band structure at 1500 cm^{-1} is quite consistent with the formation of a chelate ring with a conjugated C····O. The spectra in Fig. 6 also indicate that even after exhaustive lyopholization some water remains in Ni · H_2Ahp (but not in H_2Ahp), as indicated by the bands at 3400 and 650 cm^{-1} in the Ni · H_2Ahp spectrum. It seems likely that water molecules may be coordinated with Ni^{2+} along the axis perpendicular to the plane of the chelate shown in Fig. 10. Further evidence that one of the carbonyl functions in H_2Ahp takes part in the interaction with Ni^{2+} is the overall reduction in the relative intensity of the C=O vibration (1650 cm^{-1} region) in the Ni · H_2Ahp spectrum compared to the H_2Ahp spectrum (Fig. 6). This significant reduction in

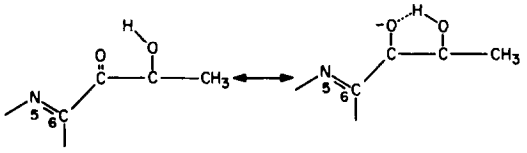

FIG. 11. Intramolecular H bonding in sepiapterin.

relative intensity occurs even though the water associated with Ni · H_2Ahp should contribute to the absorbance in the 1600-cm^{-1} region and one can readily conclude that the addition of Ni^{2+} has changed one of the carbonyl functions from the H_2Ahp molecule. The emergence of the band at 673 cm^{-1} in the Ni · H_2Ahp spectrum may also be indicative of chelate formation because Uneo and Mertel (*22*) have attributed bands in this region to metal-ligand stretching vibrations in ketoimine complexes with metal ions. The structure of the Ni · H_2Ahp complex is based on the spectral data presented here. Attempts to crystallize this complex or free H_2Ahp have been made but were unsuccessful except for small needles that were too small for use in x-ray crystallography.

Hydrogen bonding within sepiapterin (Fig. 11) appears to be consistent with the infrared spectra in Fig. 7 which show the maximum OH stretching vibration for sepiapterin at approximately 3300 cm^{-1}. This vibration occurs at a lower energy than that at 3500 cm^{-1} which is due to the water associated with the Ni^{2+} in the mixture of sepiapterin and Ni^{2+}. This shift of the OH stretch to lower energy is consistent with hydrogen bonding. Another indication of hydrogen bonding is a shift of the OH bending vibration to higher energy. Thus, an assignment of the broad band at 1370 cm^{-1} in the sepiapterin $\pm Ni^{2+}$ spectra to the OH bending vibration would be consistent with such hydrogen bonding.

The chemical similarity between the pyrimododiazepine H_2Ahp and the benzodiazepines with pharmacological activity resides in the diazapine structure for the most part. Certain benzodiazepines bind to specific receptors that are found in the mammalian brain (*23*). The possibility that 6-acetyldihydrohomopterin would interact at the Group I and Group II benzodiazepine receptor(s) was examined in collaboration with S. H. Snynder (Johns Hopkins University). No activity or inhibition of benzodiazopine binding was observed that could be attributed to H_2Ahp (unpublished results).

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