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^{1}H-NMR STUDIES WITH TETRAHYDROBIOPTERINS, EVIDENCE FOR THE STRUCTURE OF 6-PYRUVOYLTETRAHYDROPTERIN, AN INTERMEDIATE IN THE BIOSYNTHESIS OF TETRAHYDROBIOPTERIN

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Introduction

Recent reports on the biosynthesis of tetrahydrobiopterin (BH_4) have led to the recognition of 6-pyruvoyl tetrahydropterin (PPH_4) as an intermediate of key importance (1-7). Its exact chemical structure has long resisted elucidation; this was partly due to its instability and to the difficulties in obtaining substantial quantities of material. In fact, the only known procedure for the preparation of PPH_4 requires purified PPH_4-synthase (PPH_4S) and purified dihydroneopterin triphosphate (NH_2TP), the latter also having to be prepared enzymatically from GTP (8). Although the body of evidence presented in favour of the 6-pyruvoyl tetrahydropterin structure is convincing, it is mainly of secondary nature. In view of this, we consider it important to obtain further information about this intermediate's chemical properties and constitution. In a preliminary report at the 1986-meeting on "Chemistry and Biology of Pteridines", we presented ^{1}H-NMR experiments designed to follow directly the PPH_4S catalyzed conversion of NH_2TP in the NMR-tube (7). These data could correlate the disappearance of the NH_2TP signals with the appearance of a new signal which was attributed to the (3')-CH_3 group of PPH_4 (7).
Unfortunately, the signals of the hydrogens at position 7 of the pterin nucleus of PPH_4 could neither be assigned nor identified. This was due to the relatively low signal to noise ratio, as well as to the interference of

impurities. The C(7)-H_2 signals are, however, important for determining the mode of attachment of the side chain and will reflect the oxidation state of the pterin nucleus: In the case of a dihydropterin, a singlet at 4 to 4.5 ppm is expected, while a tetrahydropterin carrying a substituent at C(6) should yield an AB system centered at 3 to 3.7 ppm. In order to clarify these points, we have prepared some model tetrahydropterins, and compared their ^{1}H-NMR spectra with those of the intermediate in question. A suitable model for PPH_4 would be a tetrahydropterin with a carbonyl at C(1') such as 6-lactoyl tetrahydropterin (dihydrosepiapterin). In addition, the conversion of NH_2TP was reexamined under improved conditions, which allowed the identification of the C(7)-methylene signals.

Results and Discussion

^{1}H-NMR spectra of pterin model compounds

An ample literature exists on ^{1}H-NMR spectra of pterins and biopterins at different redox states (9-15). Unfortunately, however, with a single exception (16), we are not aware of reports on ^{1}H-NMR investigations of tetrahydrobiopterins in aqeous medium in the neutral pH region, the latter conditions being essential for kinetic and structural studies involving the use of enzymes. The choice of other solvents in the previous studies might have been dictated by solubility problems, and/or by the relative instability of neutral tetrahydropterins towards oxygen. As pointed out in the introduction, an AB signal should be expected for the C(7)-H_2 of tetrahydropterins due to the asymmetric C(6) center. This signal might be further complicated when the side chain also carries further centers of asymmetry, or if cyclisation with the nucleus occured.

The spectrum of (6R)-BH_4 at pH ~7 illustrates these problems (Fig. 1). Its interpretation is not trivial since all but the (3')-CH_3 signals of BH_4 appear in the region 3 to 4 ppm. The multiplet assigned to C(2')-H (octet) can easily be identified at 3.9 ppm by decoupling the (3')-CH_3 signal. The remaining signals are difficult to be attributed directly, although the signal at 3.2 ppm might be that of C(6)-H. We have therefore resorted to generation of selectively deuterated tetrahydropterins.

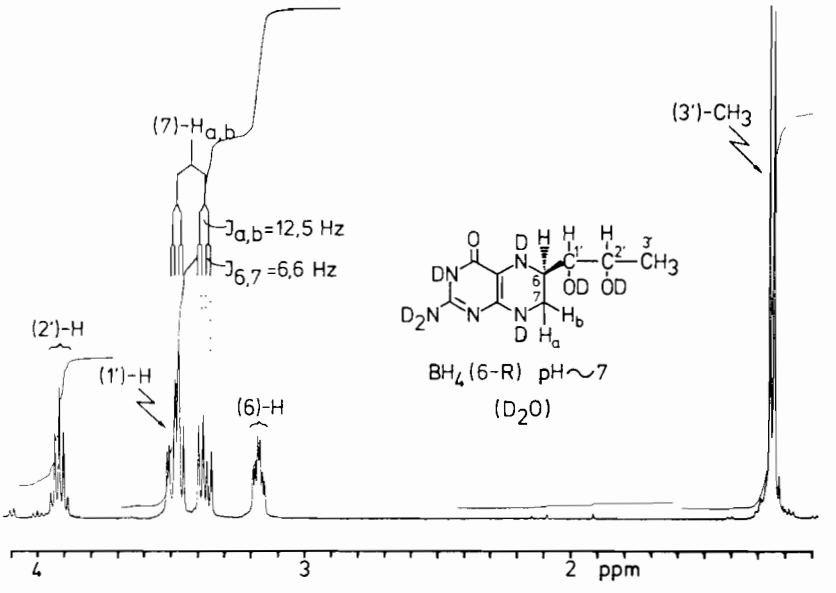

Fig. 1: ^{1}H-NMR spectrum of 6-R-tetrahydrobiopterin (BH_4). Approx. 3 mg of BH_4 hydrochloride were dissolved in ~0.4 ml D_2O containing 0.01 M phosphate buffer, and the pH was adjusted to ~7 with solid potassium carbonate (phosphate buffer prepared by dissolving P_2O_5 in D_2O, adjusted with NaOD to the pH value indicated, not corrected for H/D effect). The spectra were recorded with a Bruker 250 MHz fourier instrument (100 acquisitions) at 25°C, and the ppm scala was calibrated upon addition of a trace ethanol to the sample.

The spectrum of dihydrobiopterin (BH_2) is shown in Fig. 2 for comparative purposes.

Here, the attribution of the signals is straightforward since C(7)-H_2 is a singlet and the signals of C(1')-H and C(2')-H can be located by successive decoupling of the neighboring hydrogens.

Catalytic reduction with $^2H_2$ (Platin on asbestos in 0.1 M DCl) leads to (6-R,S),6-D-BH_4, the spectrum of which is shown in Fig. 3.

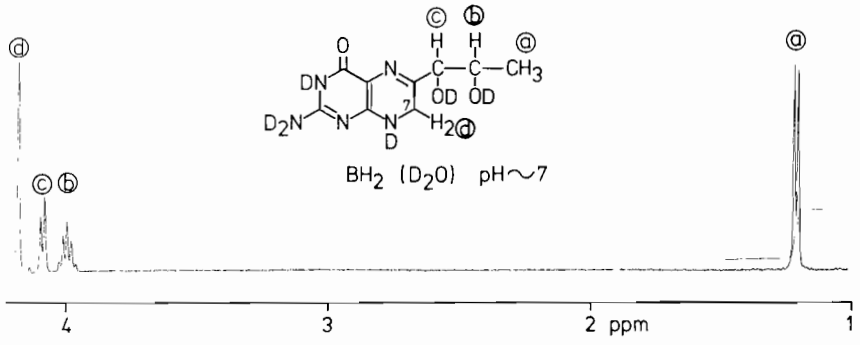

Fig. 2: ^{1}H-NMR spectrum of dihydrobiopterin. The conditions were as described in the legend of Fig. 1.

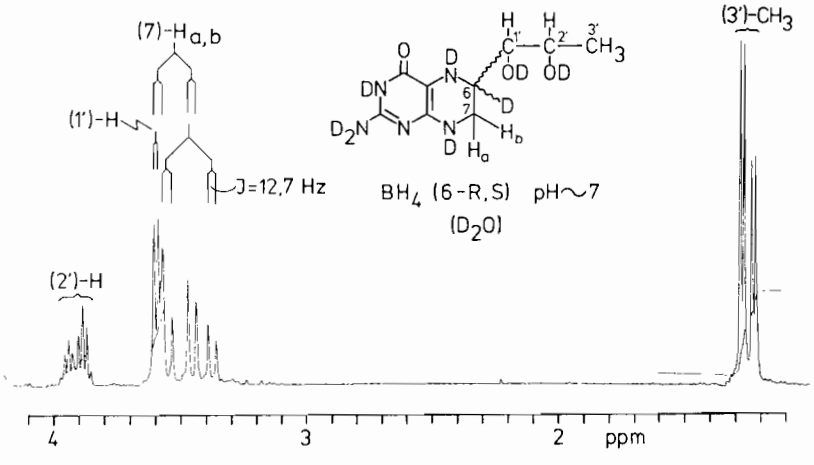

Fig. 3: ^{1}H-NMR spectrum of 6-deutero-6-(R,S)-tetrahydrobiopterin. Conditions as in the legend of Fig. 1. The compound was prepared by reduc-

tion of BH_2 with $^2H_2$ and Pt/asbestos in 0.1 N DCl. The hydrochloride obtained upon lyophilisation of the reaction mixture was then dissolved in D_2O and the pH was adjusted to a reading of 7 with NaOD. Note that comparison with the spectrum of R-BH_4 (Fig. 1) allows the facile attribution of the signal of C(6)-H of the latter and of the C(7)-H_2 AB system of S-BH_4. The signals of C(1')-H and C(2')-H can easily be assigned to the respective neighboring hydrogens by decoupling.

The first striking feature of this spectrum is the relatively large difference between the signal of the 6-R and of the 6-S-BH_4 forms (cf. two doublets of the (3')-CH_3 groups). In this case the signal of C(1')-H and of C(2')-H can be easily attributed by selective decoupling. The remaining signals must thus be those of two C(7)-H_2 AB systems belonging to the R,S pair. Again, the difference in chemical shift between the H_a and the H_b hydrogens seems to be important and is considerably different for the R form, as compared to the S form; this suggests significantly different conformations of the side chain.

The next spectrum of relevance for our purpose would be that of 6-lactoyl tetrahydropterin. This compound has been described by various groups, and can be prepared by enzymatic reduction of sepiapterin. It is, however, quite instable, and in our hands enzymatic preparation did not yield a product of sufficient quality for good ^1H-NMR spectra. Most recently, Katoh and Sueoka (16) have reported the ^1H-NMR of 6-lactoyl tetrahydropterin in neutral aqueous buffer; yet, they have neglected to show or discuss the spectral region 3 to 4 ppm, the most interesting one for our problem. We therefore decided to attempt the catalytical reduction of sepiapterin using $^2H_2$, since this would also have the advantage of leading to a 6-deuterated product. The spectrum of the starting material, sepiapterin, is simple (Fig. 4). In contrast to this, that of the product(s) was unexpectedly complex as shown in Fig. 5. In addition to some residual sepiapterin (doublet at 1.38 ppm), several signals appear below 1.0 ppm (4 doublets of different intensity). The chemical shift of these signals is typical of -(CH_2)-CH_3 groups as opposed to a -(CHOH)-CH_3 function (cf. sepiapterin, Fig. 4, or BH_4, Fig. 1, with doublets at 1.3 ppm).

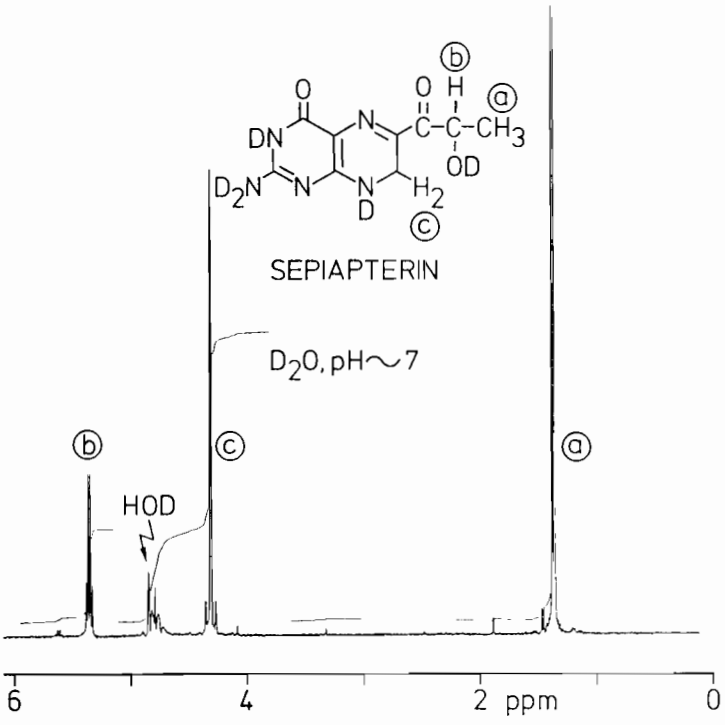

Fig. 4: ^{1}H-NMR spectrum of neutral sepiapterin. Conditions as in the experiment of Fig. 1.

Since the observed signals are doublets and not triplets, the presence of -CDH-CH_3 is most probable. In agreement with this interpretaion, the integration of the signals at 1.3 to 1.6 ppm yields 1 hydrogen, and they collapse to singlets upon decoupling the (3')-CH_3 group (Fig. 5). The chemical shift of these signals is compatible with C-H α to a carbonyl or to a hydroxymethyl function, the latter not carrying further (electronegative) substituents. The integration of the signals at 3.0 to 3.7 ppm (Fig. 5) yields 2 hydrogens (compared to 3 hydrogens for the signals at 0.9 to 1.0 ppm) which are thus attributed to the C(7)-H_2. From this follows that the C(6)-side chain of the product(s) contains 4 hydrogens and the pterin nucleus two.

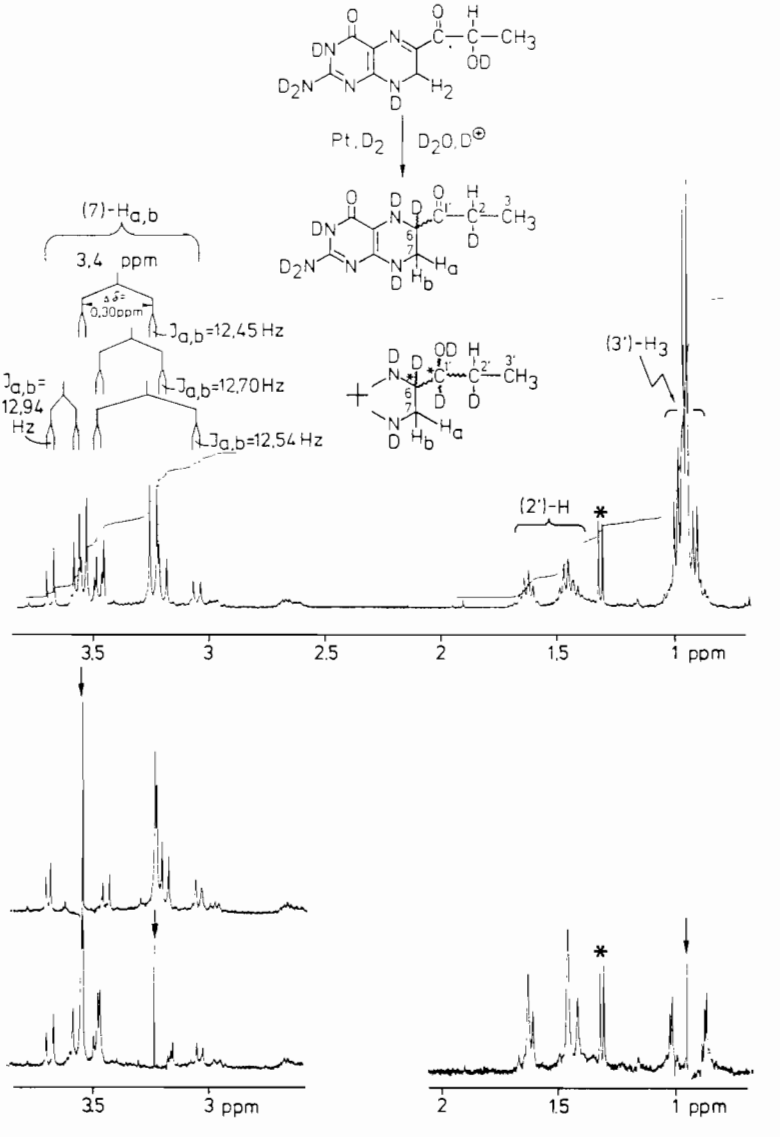

Fig. 5: ^{1}H-NMR spectrum of the mixture of products obtained upon hydrogenation of sepiapterin with $^{2}H_2$. Reduction conditions as described in the legend of Fig. 3. (*) denotes the signal of a trace unmodified sepiapterin. The lower part shows some decoupling experiments carried out in order to assign some of the signals. See text for further details.

As will be detailed below, the chemical properties and the gas chromatographic-mass spectrometric analysis of these products are consistent with this interpretation. All signals located at 3.0 to 3.7 ppm can be attributed to AB signals having different coupling constants, chemical shifts and differences in their chemical shifts of the A and B parts as shown in Fig. 5. This requires that upon or during hydrogenation, elimination of the (2')-OH and partial reduction of C(1') have occured. An attribution of the AB signals to individual components of the mixture (6-propionyl tetrahydropterin, (R/S) 6-(1'-hydroxy)propyl tetrahydropterin, cf. structures, Fig. 5) was not attempted. This would require the isolation of the pure components, a rather difficult task considering their instability. The conclusions which can be drawn from the experiments of Figs. 3 and 5 are nevertheless clear: They show the range in which to expect AB signals of tetrahydropterins C(7)-H_2 (3.0 to 3.7 ppm), the magnitude of the H_a, H_b coupling constants, and that the difference in chemical shift of H_a and H_b can vary considerably.

Analysis of products of ^2H-hydrogenation of sepiapterin

When the colourless (anaerobic) solution containing the products of hydrogenolysis of sepiapterin is exposed to air, a deep yellow colour rapidly evolves, and after several minutes a yellow precipitate formes. The UV visible absorption spectrum of this solution is similar in shape and λ_{max} to that of sepiapterin itself. This indicates that a (major) portion of the mixture is 6-propionyl tetrahydropterin which is oxidized by oxygen to (yellow) 6-propionyl-7,8-dihydropterin (2'-deoxy sepiapterin). HPLC analysis of the precipitate and of the supernatant confirm the presence of these two compounds. When the reaction mixture is analyzed by GC-MS after trimethylsilylation, three main components are found as shown in Fig. 6A. These are monodeutero-2'-deoxysepiapterin, dideutero-2'-deoxybiopterin and monodeuteropterin, the latter probably arising from (oxidative) cleavage of the side chain.

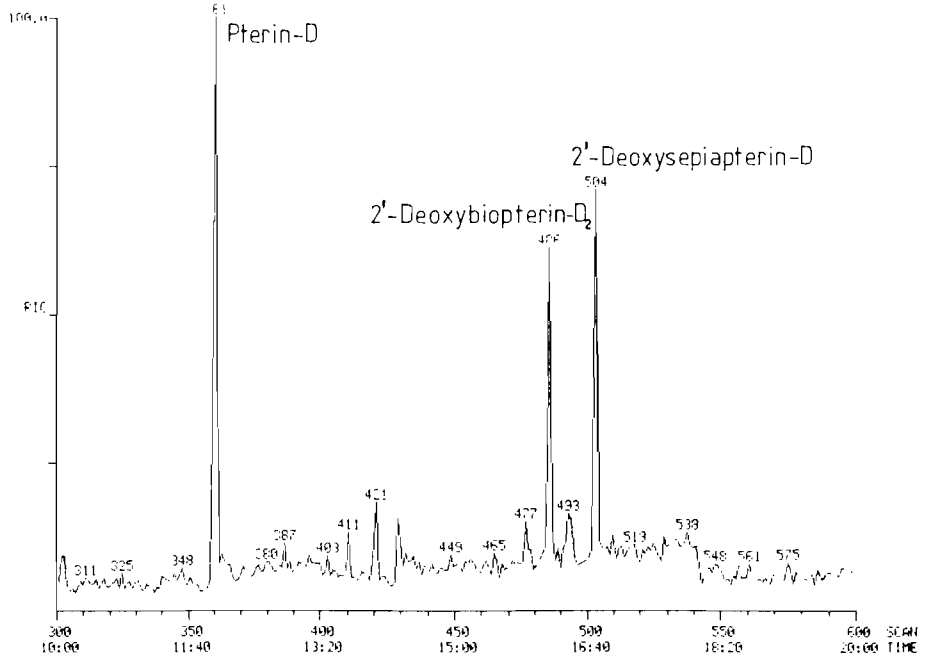

Fig. 6A: Total ion current obtained by GC-MS analysis of the silylated reaction mixture after hydrogenolysis of sepiapterin and exposure to air. These are the same products as shown in Fig. 5.

The fragmentation pattern of the 2'-deoxy biopterin (Fig. 6B) is compatible with the incorporation of a deuterium each at its position 1' and 2' (see structures on Figs. 5 and 6B). These results clearly support the structural assignments deduced from the 2^{1}H-NMR spectra discussed above.

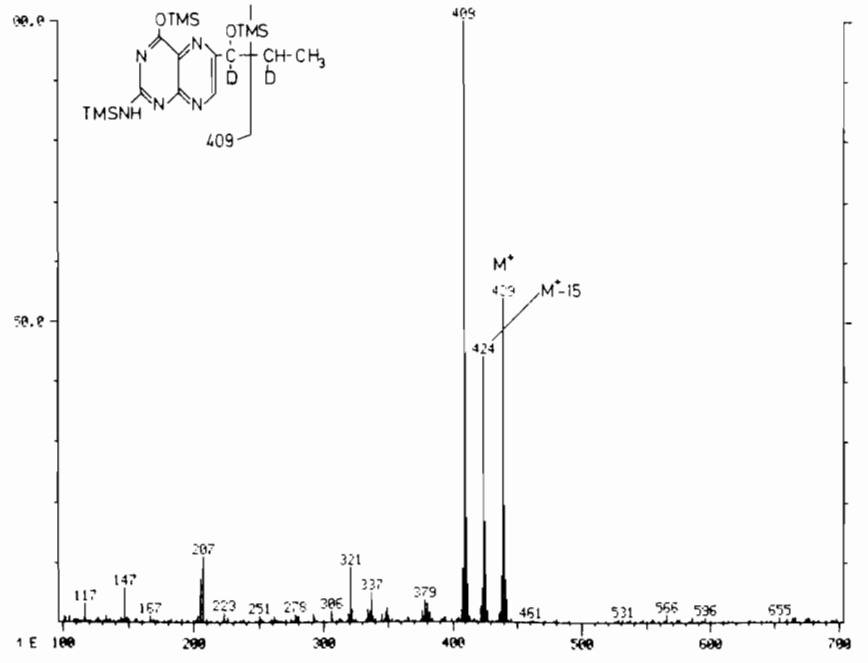

Fig. 6B: Electron impact mass spectra of dideutero-2'-deoxy biopterin TMS 3

^{1}H-NMR-spectrum of 6-pyruvoyl-tetrahydropterin (PPH_4)

Starting from NH_2TP, which was prepared enzymatically from GTP with cyclohydrolase (8) (Fig. 7, A), PPH_4 was prepared *in situ* using purified PPH_4-synthase (PPH_4S). The conditions were analogous to those described earlier (7), i.e. D_2O, pH 7.6 (adjusted to pH 7.6 without correction for H/D effect) and T = 27°C.

Fig. 7, A, shows that the starting material contains, in addition to NH_2TP, some GTP and ethanol. The latter is carried over from the preparation of NH_2TP, and was used for the calibration of the spectrum. Upon addition of PPH_4S, and after 210 min spectrum B was obtained.

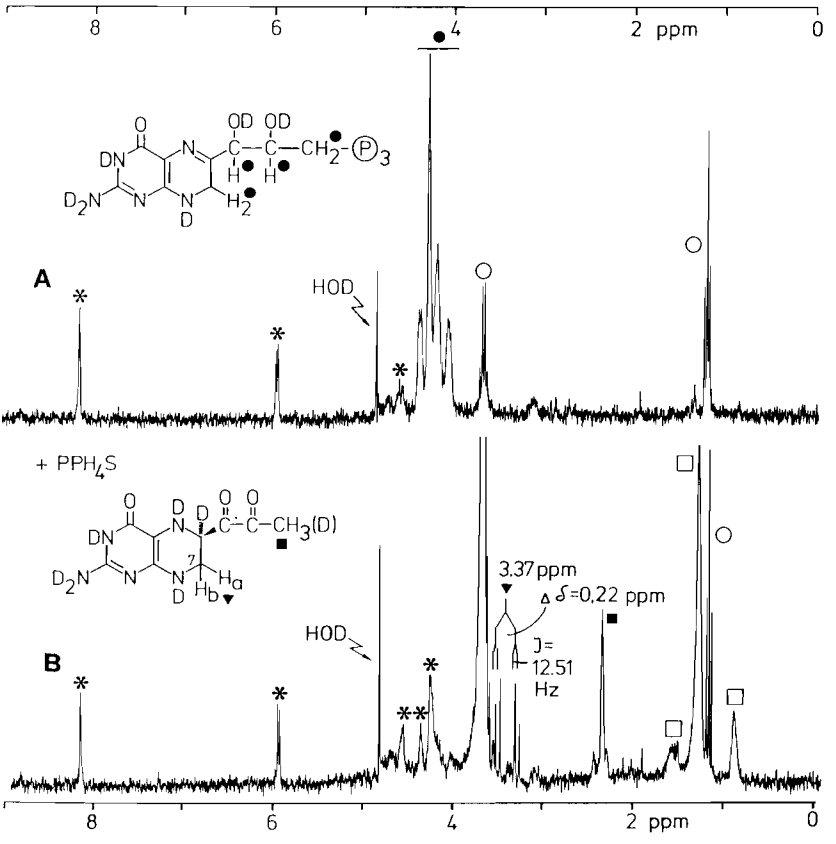

Fig. 7: ^{1}H-NMR spectrum of NH_2TP and of the product (PPH_4) obtained upon incubation with PPH_4S.
Spectrum A: solution of ~0.5 mg NH_2TP in 0.3 ml buffer containing 8 mM MgCl (200 acquisitions). Further conditions as described in the legend of Fig. 1. (*): signals of traces GTP (used for the enzymatic preparation of NH_2TP). (●): signals of the nonexchangeable hydrogens of NH_2TP (see structure insert). (○): trace ethanol (impurity from preparation of NH_2TP).
Spectrum B: same as A, but upon addition of 0.36 units PPH_4S and incubation for 210 min at 27°C. (*): as above. (□): protein signals and impurities introduced with PPH_4S. (○): as above. (■): signal attributed to the (3')-CH_3 group of PPH_4, and (▼): AB system attributed to C(7)-H_2. For further details see text.

Spectrum B gives information on several important points: GTP is essentially unchanged while new (broad) signals due to PPH_4S and to an impurity are present, which do not undergo changes during the incubation. The signals of NH_2TP disappear completely, concomitant with the appearance of two new ones (Fig. 7, B). The first of these, at 2.3 ppm, corresponds to that already observed earlier, its approximate intensity corresponds to 1-2 protons and the chemical shift is compatible with that of a methyl group α to a carbonyl. It should be noted, that this signal is relatively broad; this can be taken as suggesting the presence of different conformers or of internal cyclisation products e.g. of the side chain carbonyl group(s) with the 5- or 8-amino functions of the pterin nucleus. The relative signal intensity was estimated using the signals of GTP as standard and attributing 6 protons to the signals of NH_2TP (Fig. 7, A). Thus, the lower then expected intensity of the 2.3 ppm peak is attributed to incorporation of deuterium at position 3' during the incubation (7). In the spectral region 3.2 to 3.5 ppm a new signal is now apparent which has the shape typical for an AB system. The signal intensity, the coupling constant, and the difference in chemical shift between H_a and H_b is similar to that observed for the model compounds discussed above. It is thus reasonable to assign this signal to the C(7)-H_2 function of PPH_4. The presence of an AB signal is of importance since it proves that the neighboring C(6) atom is chiral, i.e. that the pterin must be in its tetrahydro form.

Conclusions

The present ^{1}H-NMR results have allowed to identifiy the signal of the C(7)-methylene function of PPH_4 as an AB system. This is direct and conclusive evidence for the existence of a tetrahydropterin nucleus in 6-pyruvoyl tetrahydropterin. They are compatible with formation of a (3')-CH_3 function and with removal of the C(1')-, and C(2')-hydrogens of dihydroneopterin triphosphate. The study of the NMR properties of various model pterins extends and completes those published by others. They suggest marked differences of the chemical properties between the Re and the Si side of tetrahydropterins carrying optically active substituents at C(6).

Acknowledgements

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