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BIOSYNTHESIS OF RIBOFLAVIN

^{13}C-NMR TECHNIQUES FOR THE ANALYSIS OF MULTIPLY ^{13}C-LABELED RIBOFLAVINS

PAUL J. KELLER, QUANG LE VAN and ADELBERT BACHER*
Lehrstuhl für Organische Chemie und Biochemie der Technischen Universität München, D-8046 Garching, Federal Republic of Germany

and

HEINZ G. FLOSS*†
Department of Medicinal Chemistry and Pharmacognosy, Purdue University, West Lafayette, IN 47907, U.S.A.

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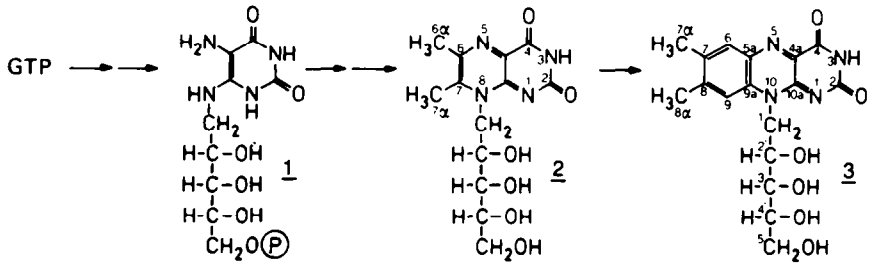
Abstract—Several recently developed NMR techniques were used to analyze samples of biosynthetically labeled riboflavin tetraacetate. The xylene ring of riboflavin consists of two biogenetically identical halves arising from dismutation of a lumazine precursor. The origin of the four carbons which eventually form this xylene ring eas investigated. Carbon-13 labeled precursors were fed to growing cultures of *Ashbya gossypii*, and the riboflavin produced was isolated and acetylated. A sample of riboflavin tetraacetate derived from [U-$^{13}C_6$]glucose gave a complex ^{13}C-NMR spectrum due to the presence of many ^{13}C–^{13}C couplings and overlapping multiplets. ^{13}C-homonuclear-J-spectroscopy, which was performed analogously to the proton experiment simplified the spectral analysis of this material. Individual ^{13}C-multiplets could be viewed, and a projection of the data onto the ^{13}C-chemical shift axis yielded a ^{13}C-NMR spectrum which was both ^{13}C- and ^{1}H- broadband decoupled (normal ^{1}H-broadband decoupling was used throughout). A ^{13}C-homonuclear-2D-chemical shift correlation spectrum was employed to determine coupling partners in the ribityl side chain. However, 2D-INADEQUATE was better suited for determining the carbon-carbon connectivities of the entire spectrum. The combination of these three techniques permitted unambiguous ^{13}C-NMR assignments to be made, as well as elucidation of the biochemically maintained ^{13}C–^{13}C connectivities in the conversion of the labeled glucose to riboflavin. A riboflavin tetraacetate sample derived from [2, 3-$^{13}C_2$]-succinate posed another analytical problem; the ovserved amounts of ^{13}C–^{13}C coupling and the calculated ^{13}C-enrichments were not the same for the corresponding carbons of the biogenetically equal halves of the xylene ring. This was found to be due to the effect of a neighboring ^{13}C-atom on the relaxation characterists of an observed ^{13}C-atom. Remeasurement of the sample in the presence of an electronic relaxation reagent and using an NOE suppressive ^{1}H-decoupling scheme, yielded equal enrichments and amounts of coupling for the biogenetically equivalent sites. The results suggest that the four carbon atoms in question arise from a pentose or its biochemical equivalent, by excision of a C-4 of a pentose rather loss of a terminal carbon atom (C-1 or C-5).

The advent of FT ^{13}C-NMR permitted the determination of the sites of biosynthetic labeling in products from ^{13}C-labeled precursors. Two major experimental approaches have been taken in order to obtain biosynthetic information by this method. The first and most straightforward approach is the feeding of singly labeled precursors followed by isolation and ^{13}C-NMR spectral examination of products. In this way one can obtain ^{13}C enrichment values for each product carbon atom, which is a measure of the efficiency of incorporation of precursor derived carbon. The second general approach involves the use of multiply ^{13}C-labeled precursors and examining the product for ^{13}C–^{13}C coupling as evidence for intact incorporation of precursor.

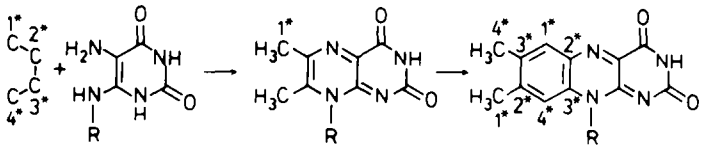
Current efforts in our laboratories involve studies on the biosynthesis of riboflavin (**3**, Scheme 1) as examined by ^{13}C-labeling analysis (for reviews of the extensive literature on riboflavin biosynthesis see Refs. 1–3). It was previously shown that the pyrimidine ring of the molecule is derived from GTP through loss of C atom 8.[4–6] Subsequent reduction of the ribose moiety and deamination of the pyrimidine ring yields 5-amino-6-(D-ribityl)amino-2,4(1H, 3H)-pyrimidinedione 5′-phosphate (**1**).[7–10] At this point 4 C atoms of unknown origin are added to **1** to give 6,7-dimethyl-8-(D-ribityl)lumazine (**2**). One molecule of **2** then donates a 4-carbon unit (C atoms 6α, 6, 7 and 7α of the lumazine) to a second molecule of **2** to form riboflavin.[1,2,11] Considerable efforts were made to determine the origin of the 4 C atoms required for the formation of **2** from **1**. Early studies by Plaut and Broberg[12,13] on the incorporation of a variety of ^{14}C-labeled precursors into riboflavin showed that acetate was not likely to be a direct precursor of the xylene ring. On the basis of subsequent studies, various authors proposed diacetyl,[14] acetoin,[15] a tetrose,[16,17] a pentose,[18] or a hexose[19] as the ultimate precursor. Recently, it has been suggested that the pyrazine ring of **2** arises through the donation of the ribityl moiety of **1** to a second molecule of **1** to give **2**.[20,21] This hypothesis is formally analogous to the known formation of **3** from **2**.

The work of Plaut[12,13] on the incorporation of ^{14}C-labeled precursors showed that both

†Present address: Department of Chemistry, Ohio State University, Columbus, OH 43210, U.S.A.



Scheme 1. Biosynthesis of riboflavin.



Scheme 2. The 4-carbon units in riboflavin biosynthesis.

D-[1-$^{14}C_1$]glucose and D-[6-$^{14}C_1$]glucose label the 4-C unit at one or both of its outer C atoms, i.e. C-7α, C-8α, C-6 and C-9 of riboflavin. One or both of the inner Cs of the 4-C unit (C-7, C-8, C-5a, C-9a) were labeled from proffered [1-$^{14}C_1$]acetate. This group also determined the regiochemistry of the transformation of **2** to **3** by the enzyme, riboflavin synthase as indicated in Scheme 2.[22] For the purpose of discussion, it is convenient to introduce an arbitrary notation for the 4-C unit as C-1* through 4* as seen in Scheme 2. Recently, biosynthetic incorporation of D-[1-$^{13}C_1$]ribose into riboflavin which led to labeling of the 1* position confirmed this regiochemistry.[23] This experiment also excluded totally symmetrical precursors such as diacetyl, since the 4* positions were not labeled.

Riboflavin has some unique properties which make it an especially interesting target for biosynthetic analysis by ^{13}C-NMR. The structure includes a ribityl side chain which has been shown earlier to be derived from the ribose side chain of guanosine.[5] Thus, the hypothesis that a pentose is also the progenitor of the aromatic ring atoms can be examined by comparing the labeling patterns of the side chain and the xylene ring of **3**. The labeling pattern of the ribityl function can also give insights into carbohydrate metabolism of the organism. Another biosynthetic property of riboflavin is the pseudosymmetrical labeling which is expected for the xylene ring on the basis of the 4-C unit by the dismutation of **2**. Thus, the following pairs of C atoms of **3** are biosynthetically identical: 6 and 8α (1*), 5a and 8 (2*), 9a and 7 (3*), 9 and 7α (4*). Knowledge of this symmetry then permits an internal check of the validity and accuracy of biosynthetic data. Moreover, this property can be exploited to check the reliability of quantitative ^{13}C-NMR techniques which assess enrichments and amounts of coupling; here sp^3 carbons (7α and 8α) are biochemically equivalent to sp^2 carbons (9 and 6, respectively).

In the present work, we decided to perform ^{13}C-NMR measurements after chemical conversion of riboflavin to its 2′,3′,4′,5′-tetraacetate. The lipophilic nature of this compound and its excellent solubility in $CDCl_3$ minimize sample heating due to the ^{1}H-decoupler, and this form is less susceptible to chemical shift changes due to concentration differences. More importantly, the acetate signals can serve as an enrichment standard since these sites always contain natural abundance ^{13}C. Disadvantages of the tetraacetate are that the acetate Me signals occur in the same chemical shift region as those of C-7α and C-8α, and the signals for the ribityl carbons 2′,3′ and 4′ are more tightly clustered in the tetraacetate.

^{13}C-NMR data for both riboflavin and the tetraacetate are shown in Table 1. The signal assignments in such biosynthetic studies are of critical importance. The assignments for riboflavin, its derivatives and isoalloxazines in general have been discussed in the literature for over 10 years.[24-28] The major problems in making these assignments are the pseudosymmetry of the xylene ring and the large number of quaternary C atoms as well as the very similar properties of C atoms 2′, 3′ and 4′.

Isotopically labeled riboflavins have been synthetically prepared by several groups. Carbons 2, 4, 4a and 10a have been labeled with ^{13}C giving assignments for these signals.[25] The unusual acidity of the protons at C-7α of the lumazine **2** permitted [6,7α-$^2H_4$]riboflavin to be synthesized.[22] Yagi *et al.*[26] have prepared ^{15}N-labeled riboflavins leading to assignments in the pyrimidine ring of the molecule. [2′-$^2H_1$]- and [3′-$^2H_1$]riboflavin have been synthesized and their ^{13}C-NMR spectra measured.[29] Lhoste *et al.*[28] have examined long-range ^{1}H–^{13}C couplings and measured ^{13}C relaxation times of 3-methylriboflavin tetraacetate. In work not reported here in detail, we have compared ^{13}C-NMR spectra of biosynthetically labeled riboflavin and the tetraacetate to yield assignments on the basis of identical enrichments.

Despite considerable experimental efforts and numerous reports, ambiguities remain in the overall ^{13}C-assignment of riboflavin tetraacetate. In order to clarify these, 2D-INADEQUATE and 2D-^{13}C-homonuclear chemical shift correlation spectroscopy were used in the present work for assignment purposes. Additionally, 2D-INADEQUATE provided information on the connectivities between labeled C atoms in ^{13}C-labeled riboflavin tetraacetate.

Table 1. ^{13}C-NMR properties of riboflavin and riboflavin tetraacetate

C-Atom	Chemical shift		T_1[a]	$^1Jc\text{-}c$[b]
	Riboflavin[c]	Riboflavin tetraacetate[d]	(sec)	(Hz)
2	155.5	154.6	20	
4	159.9	159.3	27	77 (4a)[e]; 53 (10a)[e]
4a	136.8	136.0	36	77 (4)[e]
5a	134.0	134.5	11.8	67 (6)
6	130.7	132.8	0.38	67 (5a); 61 (7)
7	135.7	136.9	7.1	44 (7a); 61 (6)
7a	18.8	19.4	1.36	44 (7)
8	146.0	148.0	7.5	43 (8a); 62 (9)
8a	20.8	21.4	1.40	43 (8)
9	117.4	115.5	0.31	66 (9a); 62 (8)
9a	132.1	131.1	10.9	66 (9)
10a	150.8	150.6	14.9	53 (10a)[e]
1'	47.3	44.9	0.20	41 (2')
2'	68.8	69.3	0.44	41 (1')
3'	73.6	70.4	0.41	46 (4')
4'	72.8	68.9	0.44	42 (5'); 46 (3')
5'	63.4	61.8	0.31	42 (4')
$\underline{C}H_3CO$		20.3 - 21.0	1.36 - 1.60	
$CH_3\underline{C}O$		169.7 - 170.6	11.8 - 17.4	

[a]Data are for 3-methylriboflavin tetraacetate from Lhoste *et al.*[28]
[b]Coupling partners are indicated in brackets. These data are compiled from several biosynthetically labeled riboflavin tetraacetate samples which we have examined.
[c]In DMSO-d_6.
[d]In $CDCl_3$.
[e]Data are for 3-methylriboflavin tetraacetate from van Schagen and Müller.[27]

RESULTS

Two of the labeled precursors which were chosen for incorporation experiments were D-[U-$^{13}C_6$]glucose and [2,3-$^{13}C_2$]succinate. We here report on the NMR methodology utilized in the analysis of the riboflavins derived from these compounds.

Spectral simplification by ^{13}C-homonuclear-2D-J spectroscopy. The incorporation of uniformly labeled general metabolic precursors into molecules of biosynthetic interest can potentially be very informative. The hindrance to such studies is that the spectrum of the product is expected to be complex. Figure 1 shows the ^{1}H-decoupled ^{13}C-NMR spectra of natural abundance riboflavin and that derived from D-[U-$^{13}C_6$]glucose. The labeled sample, which has an overall enrichment of *ca* 15%, shows complex patterns in the aromatic region and in the area of the signals for the ribityl carbons 2', 3' and 4'. In an effort to simplify the spectrum, a ^{13}C-observe variant of the ^{1}H-homonuclear-2D-J experiment of Freeman and Hill[30] was implemented. The data were acquired under continuous ^{1}H-decoupling, which after 2D-Fourier transformation and rotation of the frequency data by 45° yielded a ^{13}C-NMR spectrum in which ^{13}C-chemical shift and ^{13}C–^{13}C coupling information were represented in orthogonal frequency dimensions. Projection of the absolute value data onto the chemical shift axis then produced a ^{13}C-NMR spectrum which was both ^{1}H- and ^{13}C-"broadband decoupled" (Figs. 1 and 2). Slice plots of the 2D-spectrum gave ^{13}C–^{13}C coupling information for each C atom separately.

Effects of second order ^{13}C–^{13}C coupling can be seen in the projection of the aromatic region (Fig. 2). The natural abundance sample gives five signals in this region, however, seven peaks are seen in the projection spectrum of the labeled material. Additionally, it was anticipated that C-4a should give a low intensity signal due to its biosynthetic derivation from glycine which is present in the culture medium used in the feeding experiments. The explanation for the presence of three more signals than initially expected is that C-6 and C-5a form a strongly coupled system. The midpoints of the asymmetric doublets for each of these carbons are not at the same frequencies as the non-coupled signals. Thus, peaks b and c represent coupled and non-coupled species from C-5a, while peaks f and e are the corresponding species for C-6. A combination line arises as an artifact from the strongly coupled system and ap-

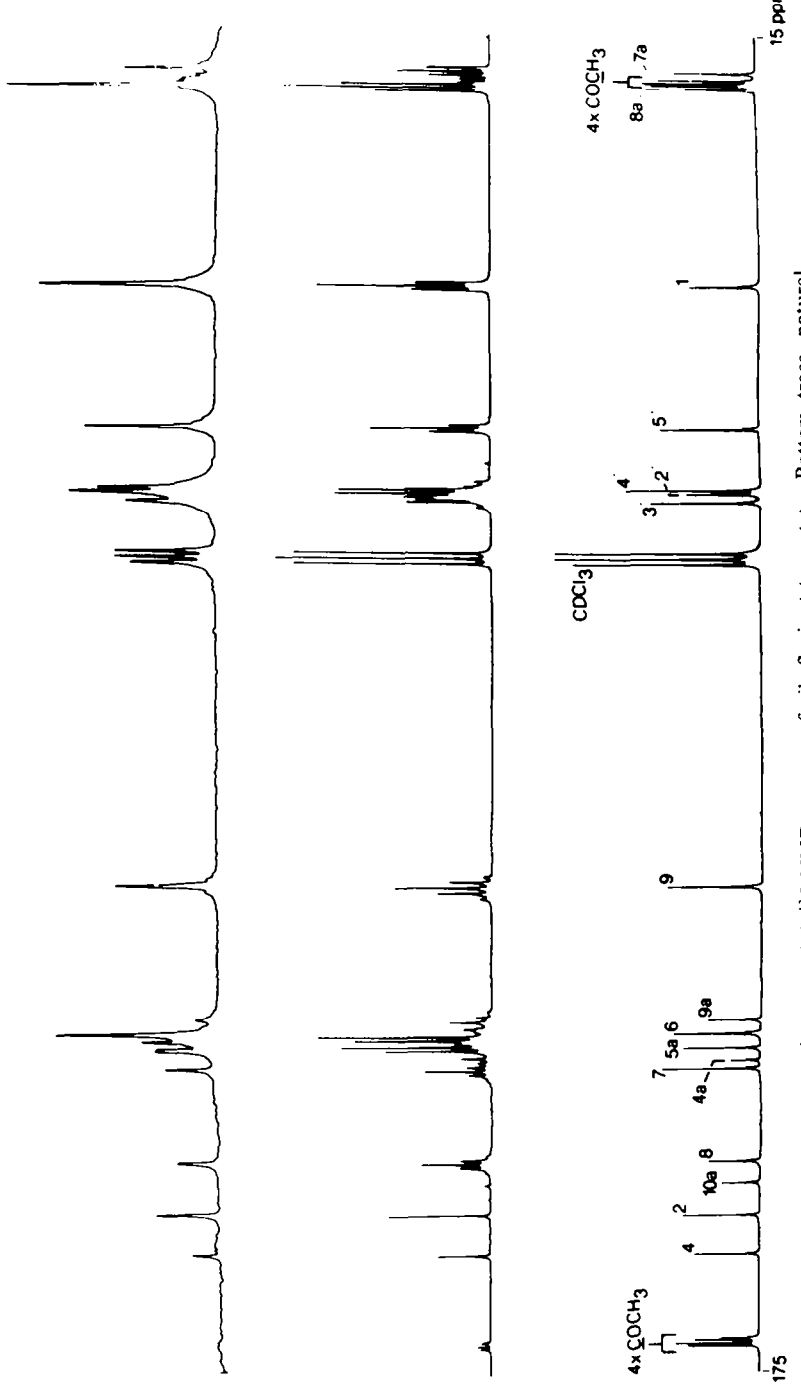


Fig. 1. 50.3 MHz ^{1}H-decoupled ^{13}C-NMR spectra of riboflavin tetraacetate. Bottom trace, natural abundance sample. Middle trace, sample biosynthesized from D-[U-$^{13}C_d$]glucose. Top trace, ^{1}H and ^{13}C-"broadband decoupled" spectrum of the labeled sample obtained by projection of a ^{13}C-homonuclear-2D-J spectrum.

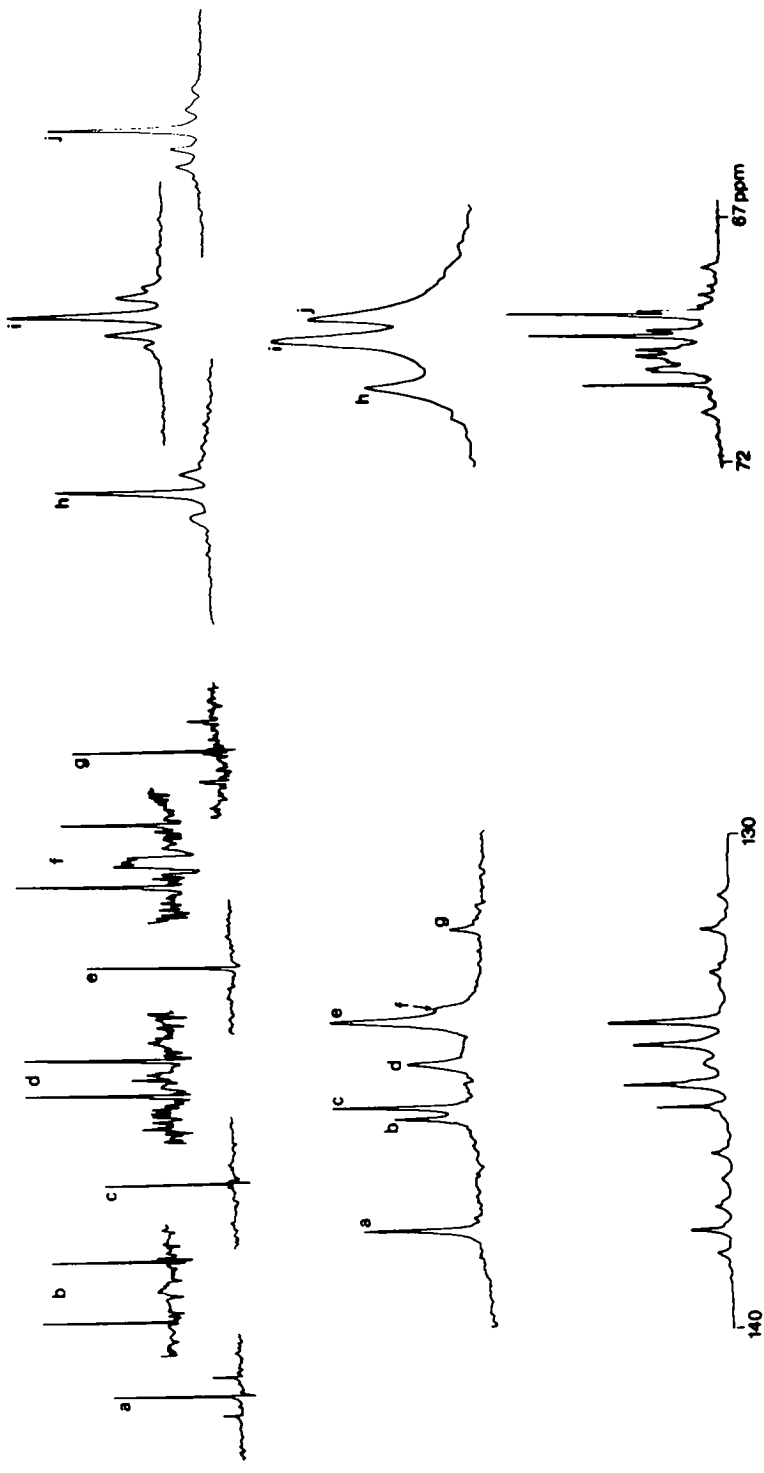


Fig. 2. ^{13}C-homonuclear-2D-J spectra of riboflavin labeled from D-[U-$^{13}C_6$]glucose. Shown are the complex aromatic (left) and ribityl carbon (right) regions as viewed in slice plots for individual carbons (top traces) and as ^{1}H- and ^{13}C-"broadband decoupled" projections (middle traces). The normal ^{1}H-decoupled ^{13}C-NMR spectra of these regions are given for comparison (bottom traces). Signal assignments are: a = C-7, b and c = C-5a, d = combination line, e and f = C-6, g = C-9a, h = C-3′, i = C-2′ and j = C-4′.

pears as peak d, the slice plot of which depicts the frequency difference between the inner lines of the AB system. The slice plots of this region are presented in phase sensitive mode.

In the region of the ribityl carbons 2′, 3′ and 4′ the projection revealed the expected number of signals. The slice plots for this area are shown in absolute value mode and are the result of summations of 10 Hz across the centers of the peaks in the chemical shift dimension. This operation was performed in order to avoid problems with off center signals arising from second-order coupling interactions, which are manifested in the projections as broadening of the peaks. It was also necessary to increase the spectral width in the coupling dimension in order to gain sensitivity as discussed below.

The T_1 relaxation times for the carbons of 3-methylriboflavin tetraacetate in $CDCl_3$ have been reported[28] and vary between *ca* 40 and 0.2 sec (Table 2). Under the condition of motional narrowing which is expected in this system, T_1 approximately equals T_2. Since a spin echo pulse sequence ($90°$—$\tau/2$—$180°$—$\tau/2$—acquisition) is used to generate the 2D data set, it follows that relaxation times which are on the same order or shorter than the average delay time τ, lead to decreased sensitivity. The maximum length of τ is dictated by the Nyquist criterion: $\tau_{max} = N/2\,sw$, where N is the number of data points (here increments) and sw is the spectral width in the second dimension. As in one-dimensional NMR spectroscopy, one may obtain increased sensitivity at the expense of resolution and *vice versa*. In the present case, increasing the sensitivity for fast relaxing carbons requires decreasing the number of sampling increments or increasing the sweep width in the second dimension.

A second relaxation related problem was encountered with this technique. It was initially expected that it would be possible to determine ^{13}C-enrichment values in the crowded regions through comparison of the integrals over the projections obtained by this technique for both the labeled and natural abundance samples. This appeared to be successful at least on a semi-quantitative basis for the ribityl C signals. However, in the aromatic region, the values obtained were found to be very unreliable. This is probably due to the effect of an enriched neighboring C on the relaxation characteristics of an observed C. Since for dipolar relaxation $1/T_1$ is proportional to $\Sigma\,\gamma_c^2\gamma_i^2 r_{ci}^{-6}$ for an observed C c which is relaxed by nuclei i, where gamma is the gyromagnetic ratio and r_{ci} is the internuclear distance, this change in relaxation is

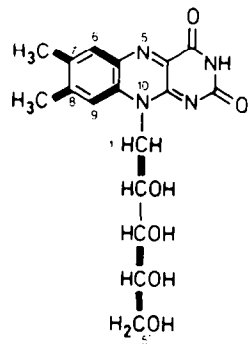
Table 2. Labeling of riboflavin tetraacetate from [2,3-$^{13}C_2$]succinate. The terms "apparent" and "actual" refer to data acquired without and with relaxation agent

C-Atom	Enrichment[a]		Coupling[b]		Coupling partners
	Apparent	Actual	Apparent	Actual	
2	2.4	2.7			
4	1.7	2.0			
4a	1.0	1.2			
5a (2*)	13.3	7.2	54	32	6
6 (1*)	9.0	8.5	26	29	5a
7 (3*)	4.1	3.7	19, 14	24	7a, 6 and/or 8
7a (4*)	8.0	7.7	6	13	7
8 (2*)	6.8	7.6	27, 8	30, 7	8a, 9 and/or 7
8a (1*)	9.0	8.4	28	30	8
9 (4*)	8.5	7.4	14	13	9a and/or 8
9a (3*)	6.8	4.0	< 5	15	9 and/or 5a
10a	0.9	1.0			
1′	7.8	6.9	21	24	2′
2′	6.2	nd[c]	30	nd	1′
3′	3.6	3.5	28	33	4′
4′	7.2	nd	39, 15	nd	3′, 5′
5′	7.2	6.2	45	44	4′
$\underline{CH_3}CO$	1.0[d,e]	1.0[d,e]			
$CH_3\underline{CO}$	0.9[e]	1.1[e]			

[a] Relative enrichment.
[b] % of the total signal integrals.
[c] Not determined.
[d] Enrichment standard.
[e] Average of the four signals.

expected to be only significant when the observed C bears no protons. These effects are discussed in more detail below.

Use of INADEQUATE for the determination of assignments and biochemical connectivities. Bax *et al.*[32] have developed a technique termed INADEQUATE for using double quantum coherence NMR to observe ^{13}C-^{13}C couplings at natural abundance while masking the much stronger signals from the non-coupled nuclei. Mackenzie *et al.*[33] and others have recently applied this technique to biochemical problems. We measured the INADEQUATE spectrum of the labeled riboflavin (Fig. 3). The pulse sequence used to generate this spectrum incorporates a delay time denoted as τ which allows for the coherence development. For weakly coupled systems, sensitivity is maximal for $\tau = 1/4Jc\text{-}c$, but it has been shown that strong coupling leads to decreased sensitivity in this experiment. This can be remedied[34] by increasing τ to $3/4Jc\text{-}c$, under which conditions sensitivity is maximal for $\Delta\nu/Jc\text{-}c = 0.85$. Significant differences between the INADEQUATE spectra of the labeled riboflavin measured under these two conditions were observed in the crowded aromatic and ribityl regions. It was also noted that the sensitivity for the satellites of C-9 was low in both cases, which is probably due to the non-uniformity of the coherence transfer across the spectrum.[35] The INADEQUATE experiment can also be implemented as a two-dimensional technique.[36] This is shown for riboflavin derived from D-[U-$^{13}C_6$]glucose in Fig. 4. ^{13}C-chemical shift is along the ordinate and double quantum frequency is on the abscissa. A mirror plane is present on the double quantum axis. In contrast to the case of natural abundance samples, for which this experiment yields signals for all of the C connectivities present in a molecule, biosynthetically labeled molecules give rise to signals which indicate intact incorporation of labeled precursors, hence signifying the biochemical carbon connectivities. We call biochemical connectivities those atomic connectivities which are retained or generated during the biochemical conversion of a precursor to a product. In the present work, the data show incorporation of 2-C units from glucose into the xylene ring as drawn in Scheme 3. This technique represents an alternative to



Scheme 3. Labeling pattern from D-[U-$^{13}C_6$]glucose.

^{13}C-homonuclear decoupling which requires specialized equipment.

A powerful attribute of 2D-INADEQUATE is that the revelation of connectivities often directly leads to assignments. If the assignments of C-7α and C-9 are accepted,[26,27] then the assignments of the entire xylene portion of the molecule are apparent. In the crowded ribityl region of the spectrum, the connectivities of C-1′ to C-2′ and C-5′ to C-4′ are indicated. At higher field, 2D-INADEQUATE of this region reveals further connectivities (Fig. 5). C-3′ shows coupling only to C-4′ and C-2′ only displays coupling to C-1′, thus indicating a break in biochemical connectivity between C-2′ and C-3′. The signals for C-4′ show how the 2D-INADEQUATE experiment responds to a 3-spin system. C-3′ and C-4′ are coupled in a large fraction of the molecules and superimposed on this coupling is the interaction of C-4′ with C-5′. Again, since the assignments of C-1′ and C-5′ are firm on the basis of chemical shift and biosynthetic arguments,[23] the displayed connectivities yield the assignments of C-2′, C-3′ and C-4′. On the basis of the break in connectivity between C-2′ and C-3′ of riboflavin from D-[U-$^{13}C_6$]glucose, it appears likely that the major portion of the proffered glucose cycled through the glycolytic pathway before conversion to ribose.

Assignments by chemical shift correlation. 2D-^{13}C-homonuclear chemical shift J-correlation spectroscopy, which was performed as described for

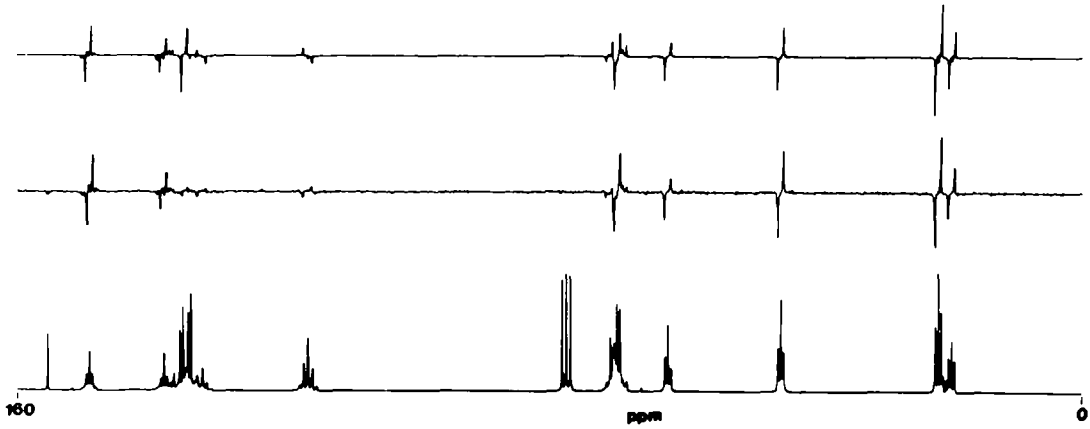


Fig. 3. INADEQUATE spectra of riboflavin tetraacetate from D-[U-$^{13}C_6$]glucose. The traces shown are for $\tau = 3/4Jc\text{-}c$ (top), $\tau = 1/4Jc\text{-}c$ (center) and the normal ^1H-decoupled ^{13}C-NMR spectrum (bottom).

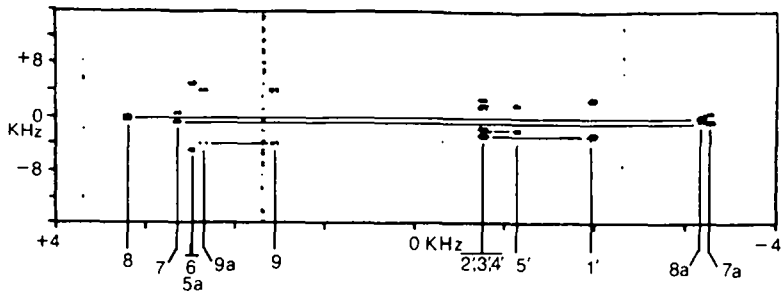


Fig. 4. 2D-INADEQUATE spectrum of riboflavin tetraacetate from D-[U-$^{13}C_6$]glucose. An artifact is visible to the left of the C-9 signal.

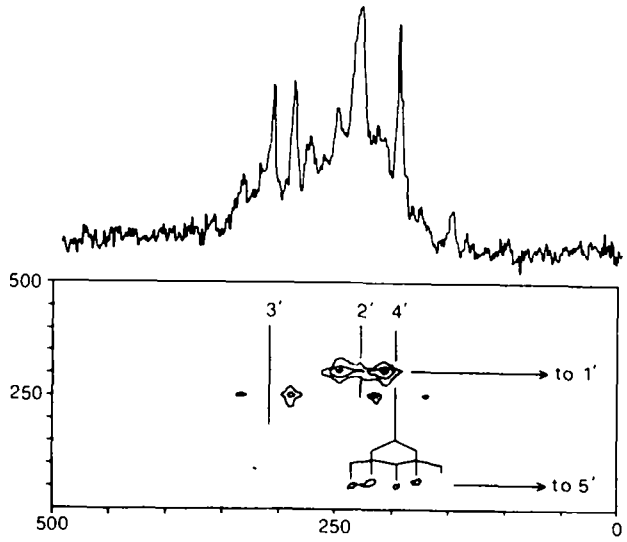


Fig. 5. 75.5 MHz 2D-INADEQUATE spectrum of the crowded ribityl region of riboflavin tetraacetate from D-[U-$^{13}C_6$]glucose. Scales are in Hz.

the proton experiment,[37] also yielded information on the crowded ribityl region (Figs. 6 and 7). Here, both axes represent ^{13}C-chemical shift. The data confirm the assignments of carbons 2′, 3′ and 4′, as well as illustrating the presence of a 3-spin system composed of C-3′, 4′ and 5′. The coupling of C-3′ to C-4′ does not show all of the weak signals that one expects in this type of spectrum, due to the second order nature of this interaction.

Quantitative determination of ^{13}C-enrichments and amounts of coupled species. As mentioned above, one of the problems encountered in the present work was that some of the quaternary carbons of riboflavin tetraacetate appeared to display different relaxation characteristics in the labeled and unlabeled samples, which hindered quantitative NMR studies. Biochemically connected carbons derived from a labeled precursor show amounts of coupling which are in excess of that expected on the basis of statistical interaction for given enrichments. Thus a neighboring ^{13}C, even at relatively low enrichment, can noticeably affect the relaxation of an observed ^{13}C. One expectation for such a system is that the coupled part of the signal for the observed C should show greater intensity for an equal number of nuclei than the non-coupled part of the signal. This would be due to the faster T_1 relaxation via dipole–dipole interaction leading to an

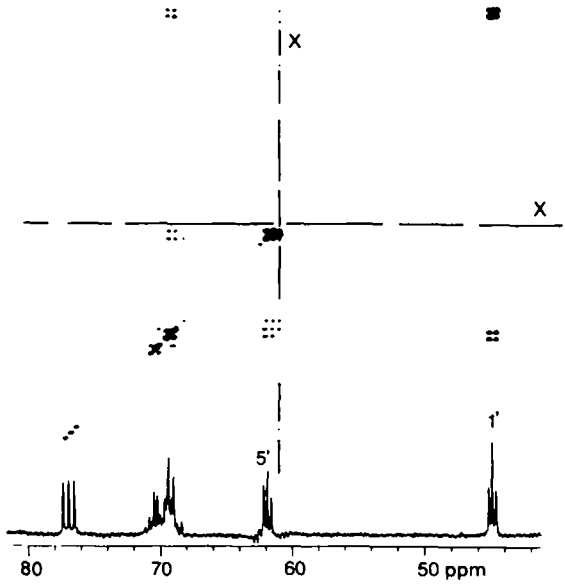


Fig. 6. ^{13}C-homonuclear-2D-chemical shift correlation spectrum of the ribityl carbon region of the riboflavin tetraacetate from D-[U-$^{13}C_6$]glucose. Artifactual points are visible along the two lines marked as "X". Folded in peaks are seen on either side of the C-5′ signal.

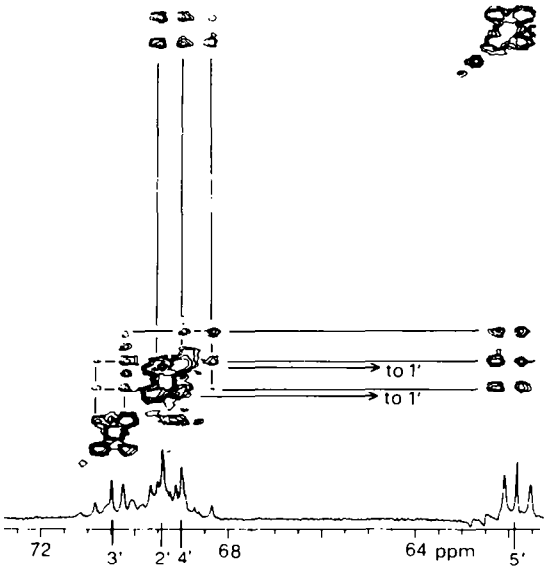
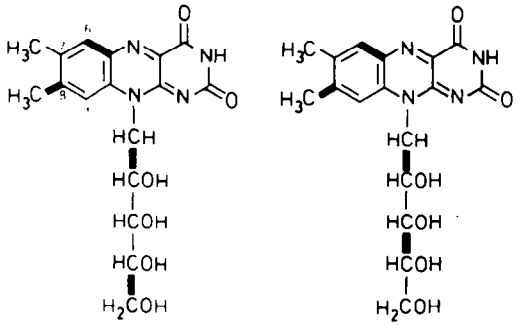


Fig. 7. ^{13}C-homonuclear-2D-chemical shift correlation spectrum of the region of C-2', 3', 4' and 5' of riboflavin tetraacetate from D-[U-$^{13}C_6$]glucose.

increased equilibrium magnetization in the detection plane for a given pulse angle and repetition rate. On the other hand, under the reasonable assumption that relaxation of these quaternary carbons is normally dominated by dipolar interactions with protons on neighboring carbons, any contribution to this relaxation from a neighboring ^{13}C would lead to a corresponding decrease in the NOE. These qualitative considerations lead to conflicting expectations for the relative intensities of the coupled vs the uncoupled signal of these quaternary carbons.

The ^1H-broadband decoupled ^{13}C-NMR spectrum of riboflavin tetraacetate which was biosynthesized from [2,3-$^{13}C_2$]succinate showed differing enrichments and amounts of coupling for some of the biosynthetically equivalent carbons in the xylene moiety (Table 2). The greatest disparity was observed for the pair C-5a and C-8 (2*), which showed enrichments of 13.2% and 6.8%, respectively. Additionally, integration over the total signals for each of these sites indicated that 54% and 27%, respectively, of the signals showed coupling to the 1* carbons. The reported T_1 relaxation times for these quaternary carbons are 11.8 sec for C-5a and 7.5 sec for C-8. Assuming that the contribution to T_1 from a neighboring ^{13}C is approximately the same for both of these carbons, the greatest change should be observed for the more slowly relaxing carbon, which is C-5a.

In order to test these ideas concerning relaxation by enriched ^{13}C-neighbors, a ^{13}C-NMR spectrum of the riboflavin derived from the labeled succinate with chromium(III) tris(acetylacetonate) was measured employing gated ^1H-decoupling (on only during acquisition) to suppress any residual NOE. The results of this measurement are shown in Table 2. The protonated carbons showed approximately the same enrichment and coupling values in both the normal ^1H-decoupled spectrum and the spectrum obtained with the relaxation reagent. However, the nonprotonated carbons of the xylene ring only reflect the biochemical symmetry in the latter experiment. It is also noted that in the former experiment the amounts of coupled species and thus also the enrichments for these carbons appeared higher than their real values, indicating that under these conditions the increased relaxation rate more than compensated for loss of NOE. The use of the relaxation reagent can potentially lead to a dynamic range problem since the solvent is also efficiently relaxed.



Scheme 4. Labeling pattern from [2,3-$^{13}C_2$]succinate.

The labeling pattern of riboflavin from [2,3-$^{13}C_2$]succinate is depicted in Scheme 4. In the ribityl side chain, it was noted that C-4' showed coupling to both C-5' and C-3', but no doubly coupled species for C-4' was observed. Thus, the labeling in this area indicates the presence of the two patterns in separate molecules. The observed connectivity of carbons 1'–2' and 4'–5' is easily explained by generation of ribose by gluconeogenesis and the pentose phosphate cycle, respectively. The labeling of C-3' and its connectivity to C-4' can be explained by assuming that a fraction of the proffered succinate cycled through the Krebs cycle before entering gluconeogenesis. The disparity in the amounts of coupling observed for the C-1' to C-2' and C-4' to C-5' connectivities permit the assessment of the relative contributions of the pentose phosphate pathway and oxidative decarboxylation of glucose in the formation of ribose as 30–50% and 50–70%, respectively.

DISCUSSION

Biosynthetic incorporation of uniformly ^{13}C-labeled precursors often leads to complex product spectra. However, recently developed NMR techniques can simplify spectral interpretation considerably. The use of ^{13}C-homonuclear-2D-J spectroscopy, which allows the determination of individual ^{13}C–^{13}C coupling patterns in overlapping regions, in combination with 2D-INADEQUATE, which yields biochemical connectivities as well as assignments, appears to be especially fruitful. Pitfalls in the quantitative evaluation of enrichments and amounts of ^{13}C–^{13}C coupling were found to be due to the effect of neighboring ^{13}C atoms on the relaxation. These problems can be overcome through the use of electronic relaxation agents and suitable instrumental parameters.

The precise origin of the 4-carbon unit which forms the xylene ring of riboflavin remains elusive, however, certain facts are known. Intuitively, the most likely precursor would appear to be diacetyl, which is a metabolite formed by many microorganisms and can react non-enzymatically with **1** to form the phosphate of **2**. However, diacetyl has been ruled out as a

precursor by the experiment with D-[1-$^{13}C_1$]ribose.[23] Acetate is also not a direct precursor as shown by work with ^{14}C-labeled acetate[12] and by unpublished experiments with ^{13}C-labeled acetate.

Carbon 1 of ribose contributes label to 1*.[23] Since C-1′ was twice as heavily labeled as the 1* positions from D-[1-$^{13}C_1$]ribose, these data do not support the proposal[20,21] that the 4-C unit arises through disproportionation of **1** in the formation of **2**. Furthermore, earlier incorporation studies with [ribose-^{14}C]guanosine demonstrated complete label retention in the ribityl side chain, but no incorporation into the isoalloxazine ring system.[5] Data obtained recently by Bresler *et al.* are also in line with these findings.[38]

It was further shown that D-[6-$^{13}C_1$]glucose labels 4*.[39] Each of the experiments with ^{13}C-labeled ribose and glucose taken separately would be in line with the incorporation of a pentose into the xylene ring. However, when taken together, we are faced with the problem that derivation of 1* through 4* from a pentose would involve elimination of an inner C atom rather than a terminal atom from the pentose.

Examination of the results from D-[U-$^{13}C_6$]glucose as seen in the 2D-INADEQUATE spectrum indicates only the biosynthetic incorporation of 2-C units into the xylene ring from glucose. However, due to the disconnection between C-2′ and C-3′ (Scheme 3), incorporation of an intact 4-C unit from a pentose into the xylene ring would not have been detected. Thus, at least superficially it would appear that incorporation into the pyrazine ring of **2** from a presumed pentose precursor must proceed through either two 2-C units or one 4-C unit, but not through a 3 + 1 or 1 + 2 + 1 combination.

The data from the incorporation of [2,3-$^{13}C_2$]succinate show that the connectivity of carbons 1′–2′ is conserved in carbons 1*–2*, whereas connectivity between 4′ and 5′ of the ribityl side chain is not reflected in 3*–4*. Although there is no positive evidence to support the conclusion, the elimination of C-4 (but not C-2 or 3) from a pentose precursor in the formation of a 4-C unit is compatible with all of the available data, however other possibilities cannot be excluded. The data from D-[U-$^{13}C_6$]glucose are reconcilable with this 3 + 1 combination by virtue of the fact that C-3′, C-4′ and C-5′ often consist of 3 contiguous ^{13}C-atoms. Thus, excision of C-4′ would give rise to the equivalent of a C-3′ to C-5′ coupled pair. At the present time, no concrete biochemical pathway for the formation of the 4-C unit can be offered, but the current experiments indicate that an unexpectedly complex mechanism may be involved. Further work is under way to help clarify the situation.

EXPERIMENTAL

Biosynthetically labeled samples of riboflavin were produced by feeding D-[U-$^{13}C_6$]glucose (90% ^{13}C, 0.25 g) or [2,3-$^{13}C_2$]succinate (90%, 0.2 g) to growing cultures of *Ashbya gossypii* as previously described,[23] which after purification and derivatization to the tetraacetate yielded 45 mg and 30 mg samples, respectively. Natural abundance riboflavin tetraacetate was prepared from commercial riboflavin. All NMR samples were dissolved in 2.5–3.0 mL of $CDCl_3$ and chemical shifts were referenced to TMS. Enrichments were calculated by comparing integrals from labeled and non-labeled samples, using the acetate methyl signals as standards. Unless otherwise indicated, NMR measurements were conducted on a Varian XL-200 spectrometer.

One-dimensional NMR spectra of D-[U-$^{13}C_6$]glucose derived and of natural abundance riboflavin tetraacetate were obtained at 50.3 MHz: pulse angle = 60°, repetition time = 2 sec, spectral width = 11 kHz, 32 K data sets. For the ^{13}C-homonuclear-J spectrum of the entire chemical shift range, the spectral width in the chemical shift dimension (sw 1) was 8 KHz collecting 4 K data sets, and in the coupling dimension the spectral width (sw 2) was 150 Hz sampled in 128 increments and zero-filled to 256 data points. For examination of the aromatic region with this technique the parameters were: sw 1 = 1300 Hz, sw 2 = 150 Hz, and in the ribityl region sw 1 = 700 Hz, sw 2 = 300 Hz; all other parameters were the same as for the entire spectrum.

1D-INADEQUATE spectra were acquired using 32 K data sets and a repetition time of 3 sec. The delay time τ was either 6 msec or 18 msec. The 50.3 MHz 2D-INADEQUATE spectrum was also acquired with a 3 sec repetition time and τ = 6 msec; other parameters were: sw 1 = 8 KHz sampled in 2 K data points and sw 2 = 8 KHz sampled in 256 increments and zero-filled to 512 data points.

The 75.5 MHz 2D-INADEQUATE of the ribityl region was acquired on a Varian XL-300 under the following parameters: Repetition time = 2 sec, τ = 18 msec, sw 1 = 3 KHz in 4 K data points, sw 2 = 1 KHz sampled in 128 increments and zero-filled to 256 data points.

The ^{13}C-homonuclear-2D-J correlated spectrum was acquired on a Nicolet NT-300 NMR spectrometer. The instrumental conditions were: sw 1 = 3100 or 1500 Hz collecting 1 K data sets, sw 2 = 3100 or 1500 Hz sampled in 256 increments and zero-filled to 512 data points.

One-dimensional ^{13}C-NMR spectra of [2,3-$^{13}C_2$]succinate derived and natural abundance riboflavin tetraacetate with and without chromium(III) tris(acetylacetonate) (0.2 M) were measured on a Bruker WP-200 spectrometer. For the samples without added relaxation agent, continuous ^{1}H-broadband decoupling was employed along with these parameters: pulse angle = 60°, repetition time = 2 sec, spectral width = 11 KHz, 32 K data sets. For the sample with relaxation reagent, inverse gated ^{1}H-decoupling (on only acquisition) was used and the other parameters were: pulse angle = 90°, repetition time = 1.2 sec, spectral width = 11 KHz, 16 K data sets.

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REFERENCES

[1]G. W. E. Plaut, *Comprehensive Biochemistry* **21**, 11 (1971).
[2]G. W. E. Plaut, C. M. Smith and W. L. Alworth, *Ann. Rev. Biochem.* **43**, 899 (1974).
[3]G. M. Brown and J. M. Williamson, *Adv. Enzymol.* **53**, 345 (1982).
[4]F. Foor and G. M. Brown, *J. Biol. Chem.* **250**, 3545 (1975).
[5]B. Mailänder and A. Bacher, *Ibid.* **251**, 3623 (1976).
[6]G. M. Shavlovskii, E. M. Logvinenko, V. E. Kashchenko, L. V. Koltun and A. E. Zakalskii, *Dokl. Akad. Nauk SSSR* **230**, 1485 (1976).
[7]R. B. Burrows and G. M. Brown, *J. Bacteriol.* **136**, 657 (1978).
[8]G. Klein and A. Bacher, *Z. Naturforsch.* **35b**, 482 (1980).
[9]E. M. Logvinenko, G. M. Shavlovskii, A. E. Zakalskii and E. Z. Senyuta, *Biokhimija* **45**, 1284 (1980).
[10]P. Nielsen and A. Bacher, *Biochim. Biophys. Acta* **662**, 312 (1981).

[11]G. W. E. Plaut and R. L. Beach, *Flavins and Flavoproteins* (Edited by T. P. Singer), p. 737. Elsevier, Amsterdam (1976).
[12]G. W. E. Plaut, *J. Biol. Chem.* **211**, 111 (1954).
[13]G. W. E. Plaut and P. L. Broberg, *Ibid.* **219**, 131 (1956).
[14]K. Bryn and F. C. Stormer, *Biochim. Biophys. Acta* **428**, 257 (1976)
[15]T. W. Goodwin and D. H. Treble, *Biochem. J.* **70**, 14P (1958).
[16]S. N. Ali and U. A. S. Al-Khalidi, *Ibid.* **98**, 182 (1966).
[17]W. L. Alworth, H. N. Baker, M. F. Winkler, A. N. Keenan, G. W. Gokel and F. L. Wood, *Biochem. Biophys. Res. Commun.* **40**, 1026 (1970).
[18]W. L. Alworth, M. F. Dove and H. N. Baker, *Biochemistry* **16**, 526 (1977).
[19]M. T. Jabasani and U. A. S. Al-Khalidi, *Int. J. Biochem.* **6**, 735 (1975).
[20]S. E. Bresler, D. A. Perumov, T. P. Chernik and E. A. Glazunov, *Genetika* **12**, 83 (1976).
[21]I. J. Hollander, J. C. Braman and G. M. Brown, *Biochem. Biophys. Res. Commun.* **94**, 515 (1980).
[22]R. L. Beach and G. W. E. Plaut, *J. Am. Chem. Soc.* **92**, 2913 (1970).
[23]A. Bacher, Q. Le Van, M. Bühler, P. J. Keller, V. Eimicke and H. G. Floss, *Ibid.* **104**, 3754 (1982).
[24]E. Breitmaier and W. Voelter, *Eur. J. Biochem.* **31**, 234 (1972).
[25]H. J. Grande, R. Gast, C. G. van Schagen, W. J. H. van Berkel and F. Müller, *Helv. Chim. Acta* **60**, 367 (1977).
[26]K. Kawano, N. Ohishi, A. T. Suzuki, Y. Kyogoku and K. Yagi, *Biochemistry* **17**, 3854 (1978).
[27]C. G. van Schagen and F. Müller, *Helv. Chim. Acta* **63**, 2187 (1980).
[28]J. M. Lhoste, V. Favqaudon, S. Ghisla and J. W. Hastings, *Flavins and Flavoproteins* (Edited by K. Yagi and T. Yamano), pp. 131–138. Japan Scientific Societies Press, Tokyo and University Park Press, Baltimore (1980).
[29]W. M. Moore and F. W. King, unpublished work; personal communication by Dr. W. M. Moore; F. W. King, Ph.D. Dissertation, Utah State University, Logan, 1973; *Diss. Abstr. Int.* **B36**, 237 (1975).
[30]R. Freeman and H. D. W. Hill, *J. Chem. Phys.* **54**, 301 (1971).
[31]F. W. Wehrli and T. Wirthlin, *Interpretation of Carbon-13 NMR Spectra*, p. 134. Heyden, New York (1976).
[32]A. Bax, R. Freeman and S. P. Kempsell, *J. Am. Chem. Soc.* **102**, 4849 (1980).
[33]N. E. Mackenzie, R. L. Baxter, A. I. Scott and P. E. Fagerness, *J. Chem. Soc.* Chem. Commun. 145 (1982).
[34]A. Bax and R. Freeman, *J. Magn. Reson.* **41**, 507 (1980).
[35]A. Bax, Personal communication.
[36]A. Bax, R. Freeman and T. A. Frenkiel, *J. Am. Chem. Soc.* **103**, 2102 (1981).
[37]A. Bax, R. Freeman and G. Morris, *J. Magn. Reson.* **42**, 164 (1981).
[38]S. E. Bresler, G. F. Gorinchuk, T. P. Chernik and D. A. Perumov, *Genetika* **14**, 2082 (1978).
[39]A. Bacher, Q. Le Van, M. Bühler, P. J. Keller and H. G. Floss, *Chemistry and Biology of Pteridines* (Edited by A. Blair). Walter de Gruyter Verlag, Berlin, in press.