Studies of Individual Amino Acid Residues of the Decapeptide Tyrocidine A by Proton Double-Resonance Difference Spectroscopy in the Correlation Mode†

William A. Gibbons,* Carl F. Beyer, Josef Dadok, Richard F. Sprecher, and Herman R. Wyssbrod*,‡

ABSTRACT: The cyclic decapeptide antibiotic tyrocidine A was studied by two relatively new methods, *viz.*, correlation proton magnetic resonance (pmr) spectroscopy and double-resonance difference pmr spectroscopy. The correlation method of spectral accumulation provided pmr spectra of good resolution, and in addition the signal-to-noise ratio achieved per unit time of accumulation was much higher than that achieved by use of the conventional continuous wave (cw) method. Furthermore, when protonated solvents are used, the correlation mode of accumulation has a distinct advantage over pulse and fast Fourier transform (fft) methods currently in use. Double-resonance difference (drd) spectra of individual amino acid residues in tyrocidine A were obtained by the correlation method when the decoupling frequency was maintained at the center frequency of the appropriate C^{α} proton multiplet and at a level of power that totally decoupled vicinal C^{α} and C^{β} protons; the resolution of these spectra was good, and the signal-to-noise ratio was high. The distinct patterns and spectral positions of the drd spectra were characteristic of the particular type of amino acid residue and, therefore, could be used as the basis for making assignments. Furthermore, the drd spectra revealed the spectral positions of individual C^{α} and C^{β} proton transitions and therefore, upon spectral analysis, could provide the chemical shifts and coupling constants of these protons. Positions of transitions were revealed even though they were hidden by overlap in the corresponding conventional single- or double-resonance spectra.

This paper may be of interest to the reader for either of two completely different reasons: firstly, because it adds to our understanding of the physicochemical properties of a particular peptide, *viz.*, tyrocidine A, and secondly, because it utilizes an experimental method[1] that may find general application in the conformational analysis of single amino acid residues within peptides by nuclear magnetic resonance (nmr) spectroscopy. Accordingly, we have divided the introduction into two separate sections: in section I, we review some of the known chemical and biological properties of the cyclic decapeptide tyrocidine A; in section II, we review difference spectroscopy in general and difference nmr spectroscopy in particular.

I. The Tyrocidines. Tyrocidine A is a member of the tyrocidine family of peptidyl antibiotics that includes gramicidin S-A as well as tyrocidines A, B, and C. Analysis of the proton magnetic resonance (pmr) spectrum of tyrocidine A by conventional double-resonance (dr)[2] techniques (Wyssbrod *et al.*, 1973,1975), internuclear double-resonance (indor) spectroscopy (Gibbons *et al.*, 1972a), pmr studies of intramolecular hydrogen bonding (Wyssbrod *et al.*, 1973,1975), and comparison with pmr parameters of gramicidin S-A (Stern *et al.*, 1968; Ohnishi and Urry, 1969) have established the backbone conformation of tyrocidine A dissolved in $(CD_3)_2SO$ to be that shown in Figure 1. Six residues of the backbone (*viz.*, L-Val[1]-L-Orn[2]-L-Leu[3] and L-Phe[6]-D-Phe[7]-L-Asn[8]) form an antiparallel β-pleated sheet structure held together by four intramolecular hydrogen bonds; the antiparallel segments are joined at each of the two ends by ten-membered hydrogen-bonded rings (β turns).

The molecule can be divided into two sides by a planar surface drawn through the backbone atoms and intramolecular hydrogen bonds. Located on one side of the molecule are two of the ten side chains, while on the other side are the other eight side chains. Of the two side chains on the "sparser" side (see Figure 1), one is aromatic (D-Phe[4]) and the other possesses the only charged group in the entire molecule (*viz.*, the cationic δ-amino group of L-Orn[2]).

In the proposed model, the C^{α} protons of L-Orn[2] and D-Phe[7] lie approximately 1.5 Å apart and are located between the innermost two intramolecular hydrogen bonds formed by the amide protons of L-Leu[3] and L-Asn[8]. (Note that the secondary rather than the primary structure is responsible for this spatial proximity.) The resonances of both of these spatially adjacent C^{α} protons are anomalously downfield in

† From The Rockefeller University, New York, New York 10021 (W.A.G., C.F.B., and H.R.W.), the Department of Chemistry, Carnegie-Mellon University, Pittsburgh, Pennsylvania 15213 (J.D. and R.F.S.), and the Department of Physiology and Biophysics, Mount Sinai Medical and Graduate Schools of The City University of New York, New York, New York 10029 (H.R.W.). *Received July 22, 1974.* Supported, in part, by National Institutes of Health Grants AM-02493 and AM-10080. The 250-MHz NMR Facility for Biomedical Research at the Carnegie-Mellon University is supported by National Institutes of Health Grant RR-00292. A preliminary report of this work was presented at the 166th National Meeting of the American Chemical Society, Chicago, Ill. (Wyssbrod *et al.*, 1973), and at the VIth International Conference on Magnetic Resonance in Biological Systems, Kandersteg, Switzerland (Wyssbrod, 1974).

‡ Recipient of National Institutes of Health Research Career Development Award K4 GM-70305. Also supported, in part, by the Life Sciences Foundation, Inc.

[1] These methods have been *pioneered* and *introduced* previously. For example, see Ernst (1966), Petersson (1970), and Dadok *et al.* (1972).

[2] Abbreviations used are: cw, continuous wave; dr, double resonance; drd, double-resonance difference; fft, fast Fourier transform; fid, free induction decay; rf, radiofrequency; sr, single resonance.

comparison with the resonances of the other eight C^α protons. These two anomalous shifts might result from the positioning of an aromatic ring over these C^α protons with the plane of the aromatic ring at an angle to the planar surface drawn through the intramolecular hydrogen bonds (Wyssbrod *et al.*, 1975).

From a chemical point of view the tyrocidines are of special interest because they may serve as useful models for testing physicochemical theories of protein structure and interaction inasmuch as they manifest self-aggregation (Battersby and Craig, 1952; Laiken *et al.*, 1969,1971; Ruttenberg *et al.*, 1965a,1966; Stern *et al.*, 1969; Williams *et al.*, 1972), bind fluorescent probes such as 8-anilino-1-naphthalenesulfonate and 2-*p*-toluidinylnaphthalene-6-sulfonate (Beyer *et al.*, 1972,1973), and paramagnetic ions.[3]

The tyrocidines are of special interest from a biological as well as from a chemical point of view. It was previously thought that the biological importance of the tyrocidines lay in their lysis of membranes by a detergent-like mode of action (Hotchkiss, 1944), but recently it has been proposed that the tyrocidines may induce sporulation of the bacterium that produces them (*viz.*, *Bacillus brevis*) by inhibition of that particular moiety of DNA-dependent RNA polymerase that is responsible for vegetative growth (Sarkar and Paulus, 1972). Because sporulation is an example of differentiation in a unicellar organism, the tyrocidines may serve as model structures for currently unidentified substances that play key regulatory roles during gestational development of higher organisms.

II. Difference Spectroscopy. A difference spectrum is generated by subtraction of one normal spectrum from another. One of these normal spectra is a control spectrum, and the other, an experimental spectrum. A daughter difference spectrum, unlike either of its parent normal spectra, reveals only those spectral transitions that have been perturbed in going from the control to the experimental condition. The control condition essentially serves to introduce a new base line against which the effect of experimentally introduced perturbations can be measured. In principle, changes in position, intensity, and width of spectral peaks can be revealed by difference spectroscopy.

Even though the nuclear magnetic resonance (nmr) spectrum of a molecule generally contains a greater amount of information than does the ultraviolet (uv) spectrum, there have been relatively few attempts to apply difference nmr spectroscopy to studies of biological molecules, whereas there have been many successful applications of difference uv spectroscopy. Among the few reported examples of the use of difference nmr spectroscopy are studies of binding sites for paramagnetic ions in proteins (Campbell *et al.*, 1973; Bradbury and Brown, 1973), oxidation and reduction of cytochromes (Gupta and Redfield, 1970a,b), the corticotropins (Patel, 1971), bovine and porcine insulin (Bak *et al.*, 1967), proton–proton exchange (spin transfer) rates and intramolecular hydrogen bonding in peptides and proteins (Bockman, 1971; Bockman *et al.*;[4] Gupta and Redfield, 1970a,b), and pH denaturation of lysozyme (King and Bradbury, 1971).

Dadok and his coworkers have given an account of obtaining single- and double-resonance pmr spectra in the correlation mode (Dadok *et al.*, 1970,1972a,b; Dadok and Sprecher, 1973,1974; Balaram *et al.*, 1973; Glickson *et al.*, 1974; also see Karplus *et al.*, 1973). A preliminary account of double-resonance difference (drd) pmr in the correlation mode has been presented (Dadok *et al.*, 1972a), and Balaram *et al.* (1972,1973) have described an intriguing use of the nuclear Overhauser effect (a form of double resonance) to study peptide–protein interactions.

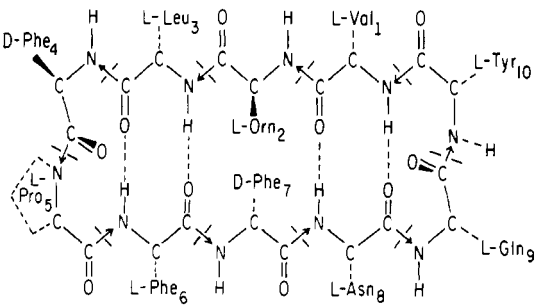

FIGURE 1: The primary structure and proposed secondary structure of tyrocidine A dissolved in $(CD_3)_2SO$. Intramolecular hydrogen bonds are denoted by - - - between H and O atoms. The backbone atoms denoted by C and N lie approximately in the plane of the page. Side-chain groups and O atoms above, in, and below the plane of the page are denoted by ◀, —, and - - -, respectively. Numbering of the amino acid residues is chosen so that residues 1–5 correspond to residues 1–5 in the related decapeptide gramicidin S-A *cyclo*(-L-Val^1-L-Orn^2-L-Leu^3-D-Phe^4-L-Pro^5-)$_2$.

It should also be mentioned that (a) Feeney and Partington (1973) obtained drd spectra at a very low "sub-tickling" level of decoupling power in order to generate a pseudo-indor spectrum of a simple molecule (1,2-dibromopropionic acid) by a pulse and fft technique and (b) Ernst (1971a,b) used the term Fourier difference spectroscopy to describe a pulse and Fourier transform technique in which the word difference refers to a difference in resonance frequencies rather than to a difference in magnitudes of *two* absorption-mode signals.

This paper represents both an extension of our previous double-resonance pmr studies and conformational analyses of individual amino acid residues in peptides and proteins (Stern *et al.*, 1968; Gibbons *et al.*, 1972a,b; Wyssbrod and Gibbons, 1973; Wyssbrod *et al.*, 1973,1975) and in the development of the use of correlation and drd nmr spectroscopy in biochemical studies (Dadok *et al.*, 1970,1972a; Dadok and Sprecher, 1973,1974). Specifically we demonstrate the application of double-resonance difference (drd) spectroscopy in the correlation mode (a) to reduce—and thereby simplify—the whole pmr spectrum of the decapeptide antibiotic tyrocidine A to the individual spectra of its constituent amino acid residues, (b) to assign the approximate chemical shifts of C^α and C^β protons in individual residues, and (c) to reveal proton transitions—and hence, by means of spectral analysis to obtain chemical shifts and coupling constants—that are hidden by overlap.

Methods

Correlation Spectroscopy. In conventional continuous wave (cw) spectroscopy, the condition of adiabatic slow passage is maintained by scanning the selected spectral range at a low rate (~1 Hz/sec), whereas in correlation spectroscopy, the condition of adiabatic rapid passage is established by scanning the range at a higher rate (~100 to ~2000 Hz/sec). As a result of rapid scanning, data can be accumulated 100–2000 times faster by the correlation than

[3] M. Fein, H. R. Wyssbrod, and W. A. Gibbons, unpublished observations.

[4] R. S. Bockman, A. G. Redfield, R. K. Gupta, and W. A. Gibbons, unpublished observations.

by the cw method. Unfortunately, the rapid scanning used in the correlation method leads to distortion of the spectrum, one manifestation of which is the well-known phenomenon termed ringing.

In order to convert the distorted spectrum obtained by rapid passage into the true spectrum, a digital filtering technique must be used. Filtering techniques were developed in the 1940's for processing communication signals in general and radar signals in particular (*e.g.*, see North, 1963). The application of one of these techniques (*viz.*, the correlation or matched-filtering process) to the processing of nmr signals was first discussed by Ernst (1966) and developed independently by Petersson (1970) and Dadok *et al.* (1972a). In essence, the correlation process consists of matching a distorted nmr spectrum (the data spectrum) with a distorted signal from a single reference peak (the reference spectrum)—*i.e.*, by the mathematical process known as correlation, the ringing reference signal can "find itself" wherever there is a ringing signal in the data spectrum (Gupta *et al.*, 1974). The correlation process *per se* regenerates signals with the familiar Lorentzian line shape characteristic of resonance signals obtained by either the cw or pulse and fft methods.

The same spectral scanning rate (Hz/sec) must apply to both the data spectrum and the reference spectrum. The reference spectrum can be either measured or calculated. For example, (a) the spectrum produced by rapid scanning of a substance such as chloroform, which produces a single pmr signal, can be used as the reference spectrum, or (b) a theoretical reference spectrum can be calculated with a digital computer inasmuch as the theoretical ringing pattern produced by rapid scanning of a single peak of Lorentzian line shape is well known.

The spectrum produced by the correlation of a data spectrum with a reference spectrum is called a correlation spectrum. Although a correlation spectrum looks similar to the corresponding undistorted cw spectrum in that the spectral peaks have a Lorentzian line shape, a closer examination of a correlation spectrum would reveal that widths of individual peaks have been doubled by the correlation process (Petersson, 1970). This does not mean, however, that the widths of all of the peaks in the correlation spectrum of a large molecule such as a peptide are twice as wide as in the corresponding cw spectrum, because peaks in such a spectrum often represent the envelope formed by summation of overlapping single peaks. Doubling of the widths of the component single peaks does not result in doubling of the width of the envelope. Indeed, the envelope is broadened by only the width of a single peak. Therefore, broadening of spectral peaks in a correlation spectrum of a complex molecule is often only slightly noticeable. In some cases, not even an isolated singlet or doublet peak appears to be broadened in a correlation spectrum. For example, in a pmr spectrum of a peptide, an amide proton doublet is relatively broad because of nuclear quadrupolar interaction from the directly bonded ^{14}N atom. Indeed, an amide proton doublet can be considered to be an envelope of closely spaced singlets whose splitting is caused by quadrupolar interaction. Thus, an amide proton doublet in a correlation spectrum appears to be only slightly broader than in the corresponding cw spectrum.

Although the peak broadening that results from the correlation process is not desirable, this slight disadvantage is easily offset by the great advantage that the correlation method provides over the cw method in increasing the rate at which data can be accumulated. Some of the savings in time can be spent in making multiple scans of the spectrum in order to improve the signal-to-noise ratio, which is proportional to the square root of the number of scans. Multiple scanning is a trivial process when a computer memory is interfaced with the spectrometer as it is with a system equipped for correlation spectroscopy.

Correlation Double-Resonance Difference Spectroscopy. Difference spectra can easily be obtained with a system in which a computer is interfaced with the spectrometer, for it is a simple matter to subtract as well as add individual scans to the spectrum storage area in the computer. For example, a double-resonance difference spectrum can be obtained (a) first by scanning the selected spectral range N times with the decoupler ("double-resonance frequency") turned off (or set far resonance) and adding each of these N single-resonance (sr) control spectra to the stored spectrum and (b) then by scanning the range N additional times with the decoupler turned on and subtracting each of these N double-resonance (dr) experimental spectra from the stored spectrum.

Double-Resonance Techniques. Drd and indor spectroscopy are different forms of a double-resonance technique that involves the use of two separate radiofrequencies designated f_1 and f_2. The sample is irradiated with both f_1 and f_2, but the receiver detects only f_1. Hence, f_1 is called the observing frequency; and f_2, the perturbing or double-irradiating frequency. When the power level of f_2 is of sufficient strength to cause total spin–spin decoupling—as in the case of drd experiments reported in this paper—f_2 is often called the decoupling frequency. In drd and pseudo-indor spectroscopy, the observing frequency f_1 is swept through the selected part of the spectrum while the perturbing frequency f_2 is kept constant, whereas in classical indor spectroscopy, f_1 is kept constant while f_2 is swept.

Instrumentation. Only one of the spectra that we report in this paper was acquired by the cw method. This 220-MHz cw spectrum (Figure 2), obtained on a Varian HR 220 field-sweep pmr spectrometer operated by a consortium at the Rockefeller University, represents a single scan made at the rate of 1 Hz/sec. The temperature of the sample was maintained at approximately 51°.

All other spectra that we report in this paper were acquired by the correlation method. These 250-MHz correlation spectra (Figures 3–8) were obtained on a frequency-sweep pmr spectrometer operated by the Facility for Biomedical Research at the Carnegie-Mellon University. Each normal single resonance (sr) spectrum (Figure 3a–c) represents the accumulation of 50 scans while each difference dr spectrum (Figures 4–8) represents the accumulation of 100 control scans with the decoupler off resonance and 100 experimental scans with the decoupler on resonance. All spectra were scanned at rates between 90 and 2000 Hz/sec. The temperature of the sample was approximately 30°.

A preliminary experiment was performed to determine the minimum level of decoupler power required to achieve total spin decoupling (Hoffman and Forsén, 1966) for our particular sample. During the accumulation of control sr spectra, the decoupler remained on at the same level of power used during the accumulation of experimental dr spectra, except that the frequency of the decoupling field was offset by 3 kHz to a position in the spectrum that was far from that of any proton resonance.

Assignment of Transitions in Pmr Spectra of Peptides. Side-chain (*i.e.*, C^{β}, C^{γ}, and C^{δ}) protons of a peptide can

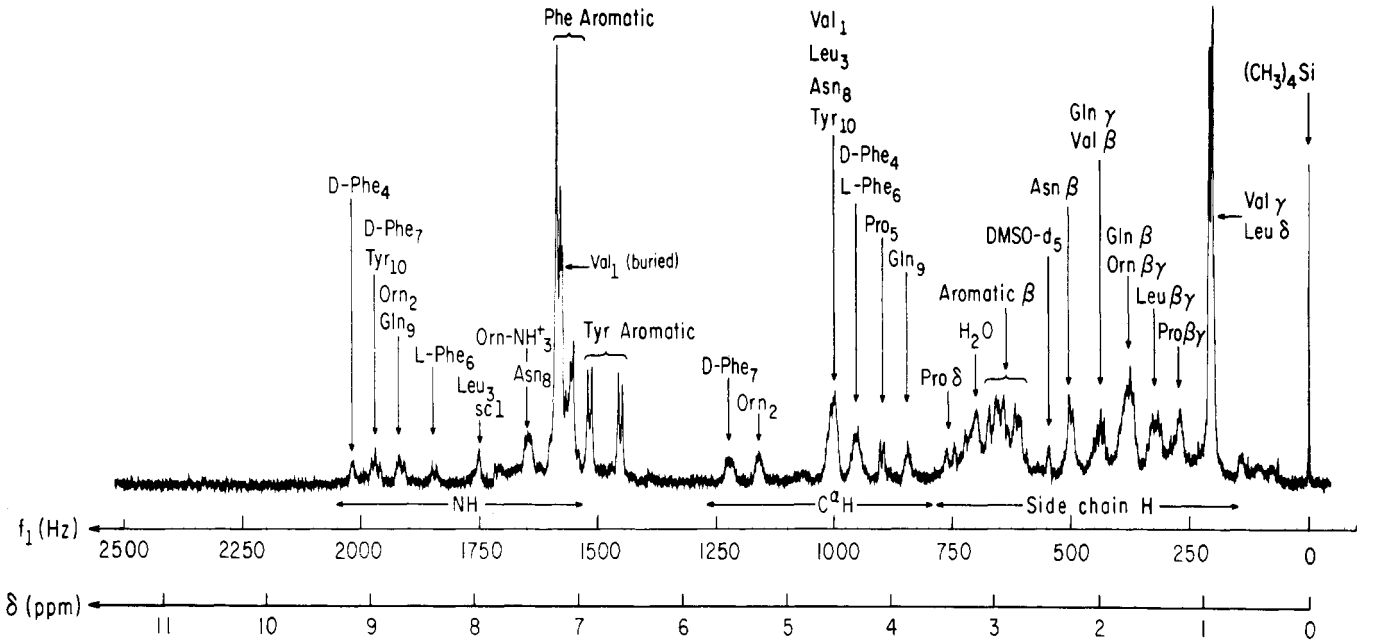

FIGURE 2: The conventional *field-sweep* 220-MHz pmr continuous wave spectrum of tyrocidine A dissolved in $(CD_3)_2SO$. The observation frequency of the equivalent *frequency-sweep* spectrum is denoted by f_1. A single scan was taken at a sweep rate of 1 Hz/sec. The temperature was 51°, and the internal standard was $(CH_3)_4Si$. DMSO-d_5 denotes dimethyl sulfoxide that contains one H and five D atoms. Side-chain carboxamide protons of Asn^8 or Gln^9 are denoted by sc. Assignments are taken from Wyssbrod *et al.* (1975).

usually be assigned with reasonable certainty on the basis of their chemical shift by comparison of the spectrum of the peptide to the spectra of the individual constituent amino acids. The same procedure, however, cannot be used to assign backbone (*i.e.*, amide and C^α) protons of a peptide because the chemical shifts of these protons seem rather dependent on conformation. Stern *et al.* (1968) pioneered the following procedure to assign amide and C^α protons in peptides. (a) The C^β protons are assigned on the basis of their chemical shift. (b) A double-resonance technique such as total spin decoupling is used to detect coupling between a C^α proton and a vicinal C^β proton in the same amino acid residue. (c) Once the C^α protons are assigned on the basis of coupling, the same procedure is used to assign amide protons inasmuch as an amide proton is coupled to the vicinal C^α proton in the same residue.

Sample Preparation. Tyrocidine A was purified by means of countercurrent distribution (Battersby and Craig, 1952; Ruttenberg *et al.*, 1965b). The sample was prepared by dissolving 70 mg (~50 μmol) of purified tyrocidine A in 0.7 ml of $(CD_3)_2SO$ (99.98% D). The internal standard was tetramethylsilane, $(CH_3)_4Si$.

Results and Discussion

In section I, we discuss some of the advantages of the correlation method over both the continuous wave (cw) method and the pulse and fast Fourier transform (fft) method for obtaining nmr spectra of peptides. In section II, we demonstrate that (a) double-resonance difference (drd) pmr spectra of a peptide can be acquired by the correlation method, (b) the drd spectra of specific protons in individual amino acid residues of the peptide can be obtained—*e.g.*, by setting the decoupling field f_2 at the resonance frequency of a specific proton, (c) the pattern and position of the individual drd spectra are characteristic of the side chain of the amino acid, (d) the characteristic nature of these individual residue drd spectra can be used as the basis for making assignments of all peaks in the whole pmr spectrum, and (e) the individual residue drd spectra reveal hidden peaks whose positions provide the basic information required to perform a spectral analysis, which in turn can serve as a basis for a conformational analysis.

I. Comparison of Various Nmr Methods: the Continuous Wave Method, the Pulse and Fast Fourier Transform Method, and the Correlation Method. A more comprehensive treatment of the correlation method may be found in Ernst (1966), Petersson (1970), Dadok *et al.* (1972a), Becker and Ferretti (1974), and Gupta *et al.* (1974).

Obtaining nmr spectra by conventional continuous wave (slow passage) spectroscopy can be a time-consuming process. For example, it required approximately 40 min to record the 2500-Hz wide continuous wave pmr spectrum of tyrocidine A in $(CD_3)_2SO$ shown in Figure 2. The signal-to-noise ratio is relatively low even though a relatively large amount (~50 μmol) of peptide was used. In order to assign spectral peaks by either conventional decoupling or indor, many extra hours must be spent in obtaining the relevant double-resonance spectra. Thus, accumulation of spectral data by some means other than the conventional continuous wave (cw) method is desirable.

Among the techniques available for increasing the rate of accumulation of nmr spectra are (a) the pulse and fast Fourier transform (fft) method (*e.g.*, see Farrar and Becker, 1971) and (b) the correlation method (Dadok *et al.* 1972a), which may also be called the matched-filter method (Petersson, 1970), the linear fast-sweep method (Dadok *et al.*, 1972a), the rapid-scan Fourier transform method (Gupta *et al.*, 1974), or the adiabatic rapid-passage method (*e.g.*, see Pople *et al.*, 1959). Unfortunately, in the pulse and fft method, a problem usually arises when nuclei capable of resonance are present in the solvent because such nuclei are excited by the pulse of radiofrequency (rf) energy and consequently contribute to the detected signal (the so-called free induction decay or fid signal). In some cases, the solvent signal can overwhelm and consequently mask the sample signal. Many attempts at removing or minimizing the

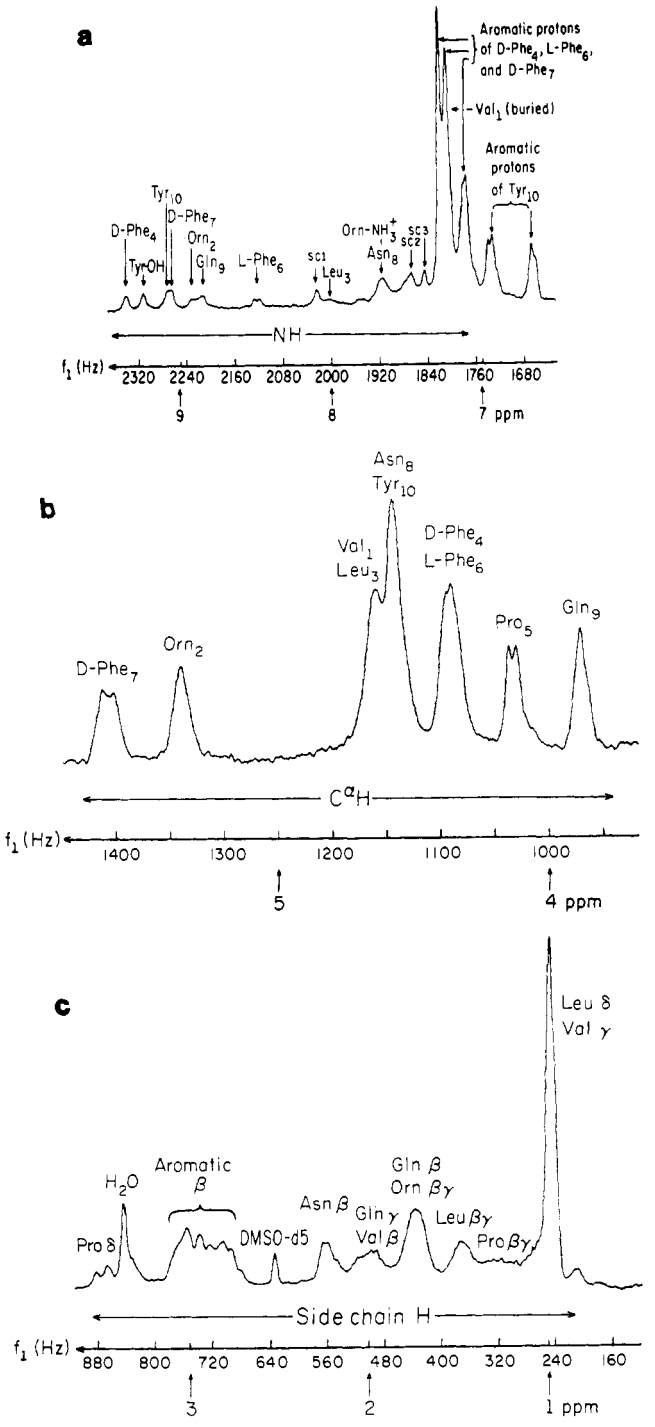

FIGURE 3: The 250-MHz correlation single-resonance spectrum of tyrocidine A dissolved in $(CD_3)_2SO$. The observation frequency is denoted by f_1; 50 scans of the entire spectrum (not shown) were taken at a sweep rate of ~2000 Hz/sec. The three spectral ranges shown (a, b, and c) were selected from the entire spectrum. Spectra a, b, and c correspond to the amide, C^α, and side-chain proton regions, respectively. Caution must be exercised in comparing spectra a, b, and c because different scales were used for both the vertical (intensity) and horizontal (frequency of chemical shift) axes of these spectra. The temperature was 30°, and the internal standard was $(CH_3)_4Si$. Side-chain carboxamide protons of Asn[8] or Gln[9] are denoted by sc; one such proton, buried under the aromatic proton resonances, is not shown. Assignments are taken from Wyssbrod *et al.* (1975).

solvent signal have been reported (*e.g.*, see Patt and Sykes, 1972; Redfield and Gupta, 1971a,b; Benz *et al.*, 1972); one particular pulse and fft system that has been reported to be relatively successful in separating proton resonance signals of a solute from those of a proton-containing solvent such as H_2O involves the modification of a commercially available spectrometer (Redfield and Gupta, 1971b). The ability of a pulse and fft system to prevent the masking of sample signals by a large background solvent signal—the so-called *dynamic range* problem—depends on the saturation level of the receiver that detects the fid signal, the precision of the analog-to-digital computer that digitizes the fid signal, and the precision of the computer (number of bits per word) used to store and process the fid signal. In the pulse and fft method, "notch filters" can be used to remove most of the solvent signal from the fid; this approach, however, can introduce phase distortion in the neighborhood of the solvent signal. In the particular case of pmr, the problem of masking arises when solvents such as H_2O or CH_3OH are used. In the correlation method—as in the slower cw method—it is possible to avoid the problem of masking simply by not scanning through the portion of the spectrum that contains the solvent signal.

In both the correlation and pulse methods, the precision of the spectrum along the chemical shift axis depends on the number of computer storage locations used to accumulate the data. The correlation method is capable, in principle, of producing a spectrum of greater precision than the pulse method, because in the former method, all of the computer memory can be dedicated to obtaining just a selected portion of the spectrum (*plus* the adjacent unwanted part of the spectrum that includes the ringing pattern that extends from the desired portion), while in the latter method, the memory must be used to obtain the entire spectrum.

Correlation pmr spectra of (a) the amide and aromatic proton region, (b) the C^α proton region, and (c) the side-chain proton region of tyrocidine A dissolved in $(CD_3)_2SO$ are shown in Figure 3a, b, and c, respectively. The entire 3000-Hz wide spectrum from which the three spectral ranges were selected and expanded represents the accumulation of 50 scans, each scan required 1.6 sec to obtain, and data processing for each range required ~1 min. Thus, the entire spectrum of tyrocidine A required only 5 min to obtain. Contrast the 5 min required to obtain the entire spectrum by the correlation method with the 40 min required by the cw method; also compare the signal-to-noise ratios of the spectra obtained by the two methods.

Thus, the correlation method is an excellent technique for quickly obtaining peptide pmr spectra with good resolution and a high signal-to-noise ratio. In the next section, we show that (a) the correlation method can be used to obtain double-resonance difference spectra of specific protons in complex molecules such as the peptide tyrocidine A and (b) the spectral information required to make assignments and perform a spectral (and subsequent conformational) analysis of each amino acid residue can be readily extracted from these difference spectra.

II. Double-Resonance Difference Spectroscopy by the Correlation Method. It is not necessary to use the correlation method to obtain double-resonance difference (drd) spectra. Use of the correlation method, however, increases the rate at which drd spectra can be accumulated.

Although use of the correlation method results in the rapid accumulation of spectral data, the dual tasks of making assignments and performing a spectral analysis are not accomplished by use of this method *per se*. In order to make assignments, a spectral perturbation technique such as double resonance must be used, and in order to perform a spectral analysis, a spectral simplification technique must

be used when individual resonances required for this analysis are obscured by overlap from other resonances. The double-resonance technique indor has been shown to be an excellent method for both assigning and simplifying complex spectra of large molecules such as peptides so that a spectral analysis can be performed (Gibbons *et al.*, 1972a,b). Unfortunately, obtaining indor spectra is a slow process because, for among other reasons, indor involves even slower scanning of the spectrum than does the single-resonance cw technique (~1 Hz/sec) discussed in the previous section.

The double-resonance difference (drd) technique, like the indor technique, can be used both to assign resonances and to simplify complex spectra so that a spectral analysis can be performed. Difference spectra are much simpler than the corresponding parent single-resonance (sr) or double-resonance (dr) spectra because they are composed of only those resonances that are perturbed by the decoupling field f_2. In essence, the positive (upward) peaks in a drd spectrum reflect the sr spectrum of specific protons in a particular individual amino acid residue, while the negative (downward) peaks reflect the dr spectrum of the same protons. To obtain the maximal amount of information by means of drd spectroscopy, it would be necessary to acquire a series of drd spectra over a wide range of decoupling power while maintaining a constant decoupler frequency. We chose, however, in this initial study to maintain the decoupling power at a constant level that was sufficient to achieve total spin decoupling.

Figure 4 shows a series of drd spectra of the C^β proton region of tyrocidine A dissolved in $(CD_3)_2SO$. In this series the decoupling frequency f_2 was incremented through the C^α proton region from 1250 to 900 Hz at 10-Hz intervals. Only a selected part of the entire series is shown in the figure. No discernible peaks appear in the uppermost spectrum because no C^α proton is perturbed when the decoupling frequency f_2 is set at 1210 Hz. As f_2 approaches 1150 Hz, however, both positive and negative peaks begin to appear, and indeed when f_2 = 1150 Hz, the maximal effect of the decoupling rf field on the C^β protons of Val^1 and Leu^3 is observed. As f_2 is decreased from 1150 Hz, the drd spectra for the C^β protons of Val^1 and Leu^3 gradually vanish, and drd spectra for other amino acid residues begin to appear. The C^β protons of a particular residue were optimally revealed in the drd spectra when the frequency of the decoupling field corresponded to the chemical shift of the C^α proton of that residue. In this particular series, drd spectra for the C^β protons of seven of the ten residues of tyrocidine A were strongly revealed. In addition, the C^β protons of an eighth residue (*viz.*, Pro^5) were weakly revealed when f_2 = 1020 Hz. More scans will be required to generate a clearer drd spectrum for the Pro^5 residue. Even then, a spectral analysis will be difficult because the proximity in chemical shift of the resonances of the two C^γ protons to those of the two C^β protons in a prolyl residue leads to the formation of a very complex non-first-order spin system.

In another series of experiments, the decoupling frequency f_2 was incremented through the C^α proton region from 1450 to 1250 Hz at 10-Hz intervals. In this particular series, a drd spectrum for the C^β protons of D-Phe^7 was strongly revealed when f_2 = 1400 Hz, and a drd spectrum for the C^β protons of Orn^2 was weakly revealed when f_2 = 1330 Hz. More scans will be required to generate a clearer drd spectrum for the Orn^2 residue. Even then, a spectral analysis will be difficult for the very same reasons that it will be difficult to analyze the drd spectrum of a prolyl residue.

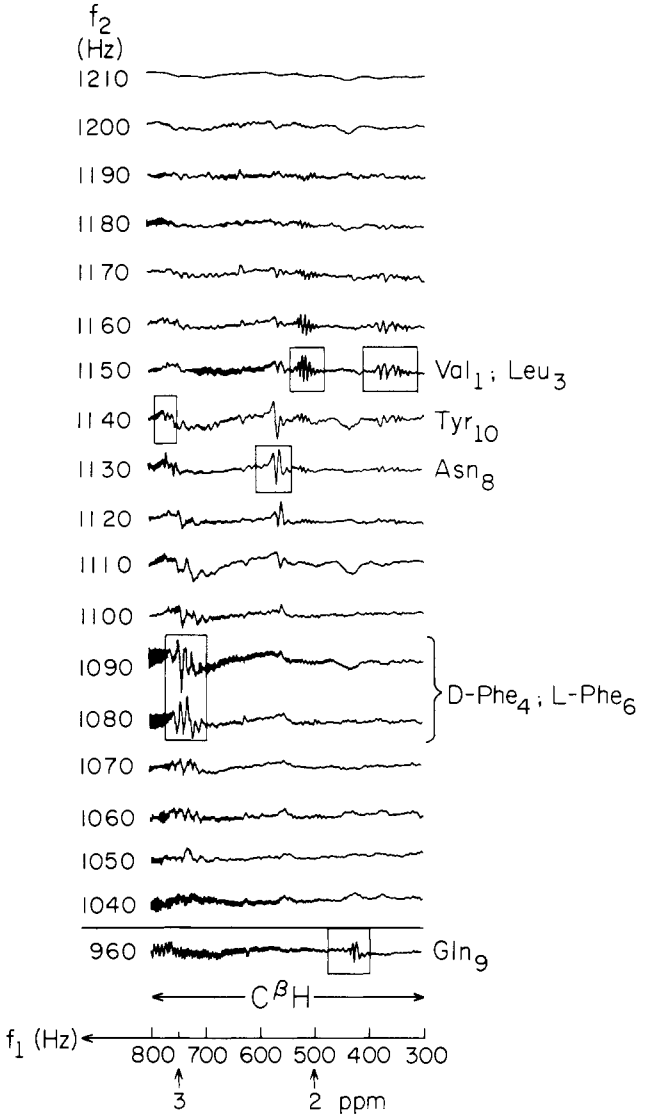

FIGURE 4: The 250-MHz correlation double-resonance difference spectrum of part of the C^β proton region of tyrocidine A dissolved in $(CD_3)_2SO$. The observation frequency is denoted by f_1, and the frequency of the decoupling field, denoted by f_2, is in the C^α proton region. Resonance signal observed when the decoupler is offset by 3 kHz from the indicated value of f_2 contribute to the generation of positive (upward) peaks, while those observed when the decoupler is set at the indicated frequency (f_2) contribute to the generation of negative (downward) peaks. Decoupling power was sufficient to achieve *total spin decoupling;* 100 scans were taken with the decoupler offset from f_2; and an additional 100 scans, with the decoupler set at f_2. The sweep rate was ~500 Hz/sec. The temperature was 30°, and the internal standard was $(CH_3)_4Si$.

Thus, in the two series of experiments described above, the C^β protons of all ten residues of tyrocidine A were revealed: eight were revealed strongly; and two, weakly. It is also clear from an examination of the drd spectra in Figure 4 that the patterns and positions of specific multiplets in the drd spectra are quite characteristic of each type of amino acid residue and could be used as the basis for making assignments. Furthermore, drd spectroscopy appears to be an excellent method for revealing individual transitions that are hidden in ordinary sr or dr spectra. The spectral positions of the hidden transitions are necessary information for performing a spectral and subsequent conformational analysis.

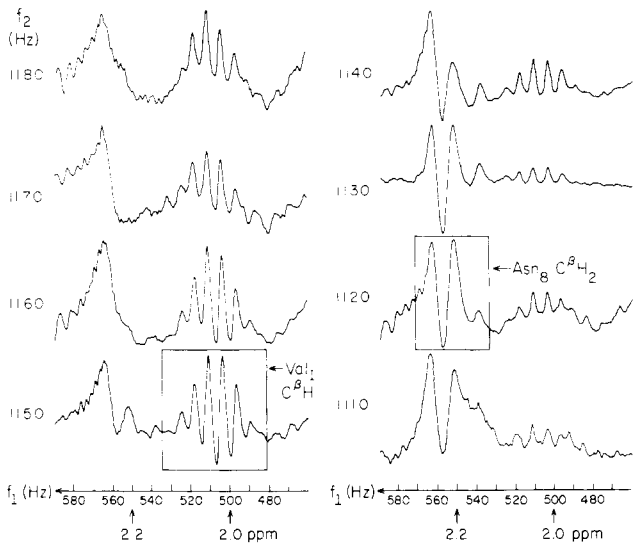

FIGURE 5: The 250-MHz correlation double-resonance difference spectrum of the C^β protons of the valyl and asparaginyl residues of tyrocidine A dissolved in $(CD_3)_2SO$. All comments in the caption of Figure 4 also apply to this figure, except the sweep rate was ~90 Hz/sec.

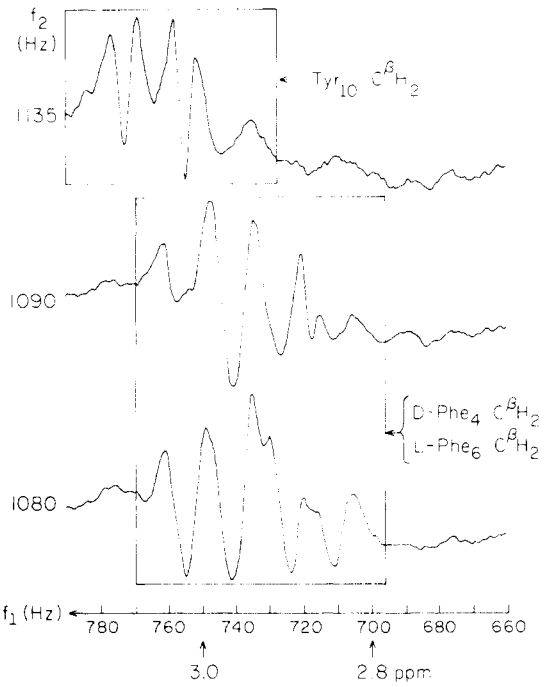

FIGURE 6: The 250-MHz correlation double-resonance difference spectrum of the C^β protons of *three* of the four aromatic residues of tyrocidine A dissolved in $(CD_3)_2SO$. See Figure 7 for the remaining aromatic residue. All comments in the caption of Figure 4 also apply to this figure, except the sweep rate was ~90 Hz/sec.

Unfortunately, the drd spectra obtained in the two series of experiments described above are not amenable to spectral analyses principally because the selected spectral sweep widths are too large to obtain the requisite precision for the location of individual transitions. Therefore, we used these spectra to obtain both the approximate location of the C^β proton resonances and the proper value of the frequency of f_2. We then selected sufficiently small sweep widths in order to obtain the expanded drd spectra shown in Figures 5–8. C^β proton resonances of Val^1 and Asn^8 are revealed in great detail in Figure 5; those of D-Phe^4, L-Phe^6, and Tyr^{10}, in Figure 6; those of D-Phe^7, in Figure 7; and those of Gln^9, in Figure 8.

The simple structure of each drd spectra shown in Figures 4–8 is readily apparent, especially when a drd spectrum is compared with the single-resonance spectrum of the corresponding region shown in Figure 3c. Note that C^γ and C^δ proton resonances overlap and thereby obscure some of the C^β proton resonances in the single-resonance spectrum, whereas they make no contribution whatsoever to the drd spectra because neither C^γ nor C^δ protons are significantly coupled to C^α protons—i.e., to those protons perturbed by f_2. In its ability to reveal hidden transitions that are obscured by overlap, the drd technique resembles the indor technique.

Let us consider a specific example to show how the drd technique can reveal hidden transitions. The resonances of the C^β proton of a valyl residue are manifest as a unique spectral pattern that results because the C^β proton is coupled to seven other protons (one C^α plus six C^γ protons). Indeed, the distinctive pattern of the valyl C^β proton multiplet can usually be recognized in an ordinary single-resonance spectrum. In the spectrum of tyrocidine A, however, the C^β proton resonances of Val^1 are partially obscured by the C^γ proton resonances of Gln^9 (see the peak centered at 2 ppm in Figure 3c). Because C^γ proton resonances do not appear in the drd spectra when C^α protons are perturbed by f_2, the C^β proton resonances of Val^1 are revealed in the drd spectra shown in Figures 4 and 5. We shall next consider why it is important to reveal hidden transitions such as those of the valyl C^β proton. This importance can be traced back to the purpose of nmr studies of peptides.

One ultimate aim of nmr studies of a peptide is to provide information on its solution conformation, which hopefully can then be related to its biological activity. Conformational parameters are directly related to nmr parameters such as coupling constants between vicinal atoms (*e.g.*, between a C^α proton and a C^β proton on the same residue). These nmr parameters must be extracted *via* a spectral analysis. A vital step in a spectral analysis is the separation of a complex spectrum into simple subspectra from which nmr parameters can be readily extracted by standard analytical procedures (for a list of some of these procedures, see Gibbons *et al.*, 1972b and Wyssbrod and Gibbons, 1973).

The validity of a spectral analysis depends, in part, on the correct grouping of individual transitions into subspectra. Correct grouping is assured by the indor technique because indor spectra provide unequivocal information for the construction of spin energy level diagrams, which in turn directly reveal subspectra. The drd technique, as well as the indor technique, can be used to provide information for the separation of a spectrum into subspectra, but great care must be taken to assure that the transitions revealed in the drd spectra are properly grouped together to form true rather than apparent subspectra. Interpretation of drd spectra are no doubt further complicated by the Bloch–Siegert effect, which is a shift in the resonance frequency that results from the vectorial addition of the magnetic field of the decoupling irradiation (f_2) to the large constant field of the external magnet—*i.e.*, complications will arise if the Bloch–Siegert shift is different for the two parent spectra that are subtracted to form a daughter drd spectrum (Bloch and Siegert, 1940; Abragam, 1961). Although indor spectra are richer in information content than drd spectra, the latter can be obtained more rapidly than the former because in the drd technique (a) the spectrum is scanned rapidly

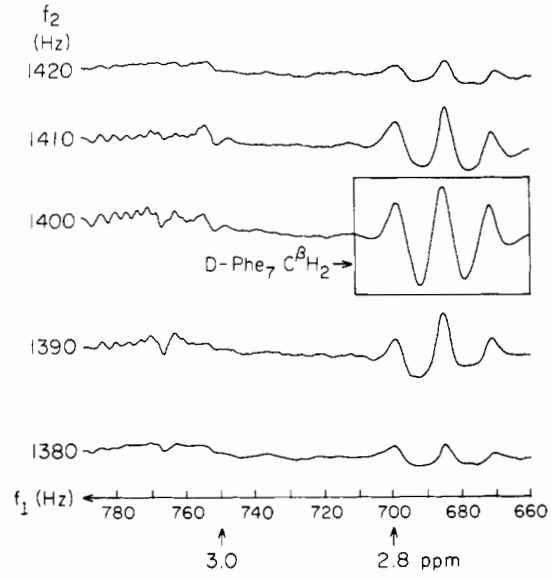

FIGURE 7: The 250-MHz correlation double-resonance difference spectrum of the C^β protons of *one* of the four aromatic residues of tyrocidine A dissolved in $(CD_3)_2SO$. See Figure 6 for the other *three* aromatic residues. All comments in the caption of Figure 4 also apply to this figure, except the sweep rate was ~90 Hz/sec.

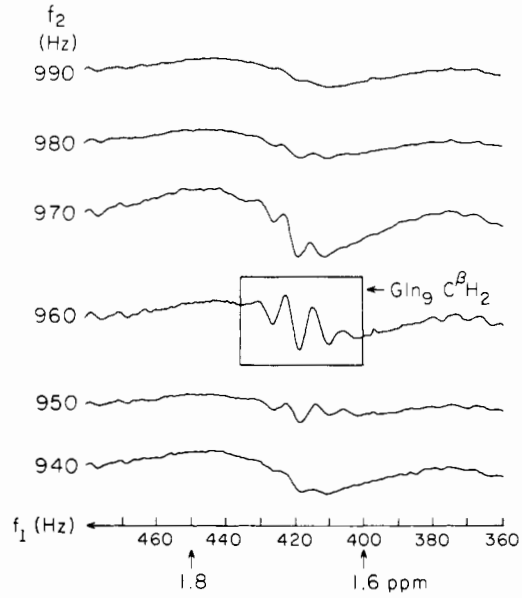

FIGURE 8: The 250-MHz correlation double-resonance difference spectrum of the C^β protons of the glutamyl residue of tyrocidine A dissolved in $(CD_3)_2SO$. All comments in the caption of Figure 4 also apply to this figure, except the sweep rate was ~90 Hz/sec.

(~100 to ~2000 Hz/sec), (b) a high decoupling level of f_2 power is used, and (c) the exact constant frequency setting of f_2 is not critical, while in the indor technique (a) the spectrum is scanned slowly (≤0.2 Hz/sec), (b) a very low sub-tickling level of f_2 power is used, and (c) the exact constant frequency setting of f_1 is quite critical. Hopefully, the advantage in speed of the drd technique will outweigh the advantage of the indor technique in providing unequivocal information on the composition of subspectra.

We used our drd spectra as the basis for the formation of subspectra from which we extracted chemical shifts (δ's) of various C^β protons and the vicinal coupling constants between various C^α and C^β protons ($^3J_{\alpha CH-\beta CH}$'s). Each value of $^3J_{\alpha CH-\beta CH}$ provides information on the dihedral angle χ_1, which in turn, either partially or totally defines the side-chain conformation of the corresponding amino acid residue. We, however, do not believe that nmr parameters extracted from drd spectra are sufficiently accurate unless computer simulation of drd spectra is used to confirm these parameters and unless the drd spectra are also determined as a function of decoupling power. Furthermore, an error analysis should be performed in order to determine the "softness" of the parameters—*i.e.*, the relationship of the error in the determination of the frequency of each of the transitions used in the analysis to the error in the determination of the nmr parameter in question. At present, we are working on the problems of (a) computer simulation of drd spectra, (b) effect of decoupling power on the generation of drd spectral patterns and peak positions, and (c) error analysis.

In this paper, we demonstrated that the drd technique can be used to reveal hidden transitions that are needed for a spectral (and subsequent conformational) analysis. We believe that reliable nmr parameters can be extracted from drd spectra, but that these parameters should be confirmed by spectral simulation and that an error analysis should be performed to ascertain the accuracy of these parameters. It may well be that the most fruitful approach to conformational analysis may combine both the drd and indor techniques: the drd technique would be used to determine rapidly the approximate location of hidden transitions, and the indor technique—or pseudo-indor technique of Feeney and Partington (1973)—would then be used to determine the exact location of these transitions and the progressiveness or regressiveness of the connections between these transitions.[5] Information on progressiveness or regressiveness is vital for the construction of spin energy level diagrams that directly reveal subspectra. Indeed, the reason that the indor—but not the drd—technique provides unequivocal information on the separation of subspectra is that positive and negative signals of indor—but not of drd—spectra are directly related to progressiveness or regressiveness, respectively. Drd spectra obtained with the decoupler power set at a very low "sub-tickling" level (such as that used to obtain indor spectra) rather than at a high "total decoupling" level (such as that used to obtain the drd spectra shown in Figures 4–8 of this paper), however, should contain the same content of information as the corresponding classical indor spectra (*e.g.*, see Feeney and Partington, 1973).

Conclusion

In this paper, we discussed the basic principles of the correlation method and the advantage of this method (a) over the conventional cw method in speeding the acquisition of spectra and (b) over current pulse and fft methods when protonated solvents are used. We showed that we could use the correlation method to obtain both normal single-resonance and double-resonance difference (drd) pmr spectra of the peptide tyrocidine A and that these correlation spectra exhibited good resolution and a high signal-to-noise ratio. We obtained drd spectra of specific C^β protons in individual

[5] If two transitions have an energy level in common, then they are either *progressively* or *regressively* connected. In a progressive connection, the common energy level lies intermediate between the two levels that are not in common, while in a regressive connection, the common energy level lies either above or below the other two levels (Kaiser, 1963; Anderson *et al.*, 1963; Kowalewski, 1969).

residues. These distinctive patterns and positions of the individual drd spectra aided in the making of assignments. Furthermore, the positions of hidden transitions necessary for a spectral analysis were revealed in the drd spectra. Drd and indor spectroscopy were compared as means for revealing the hidden spectral parameters necessary for a spectral analysis. In principle, both drd and indor spectra are capable of providing sufficient information for performing a spectral analysis. Whereas the information content of indor spectra permits a more certain spectral analysis than does that of drd spectra, drd spectra can be obtained more quickly than indor spectra. In most cases, the advantage of speed probably outweighs that of information content, and accordingly, in these cases, drd spectroscopy is the method preferred over indor spectroscopy. Furthermore, in principle, the drd technique is capable of revealing the same information content revealed by the indor technique if a very low "sub-tickling" rather than a high "total spin decoupling" level of decoupling power is used (*e.g.*, see Feeney and Partington, 1973).

Acknowledgment

The authors would like to thank Mr. Peter Ziegler for his assistance in recording the 220-MHz cw spectrum shown in Figure 2.

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Properties of Hydroxylase Systems in the Human Fetal Liver Active on Free and Sulfoconjugated Steroids†

Magnus Ingelman-Sundberg, Anders Rane, and Jan-Åke Gustafsson*

ABSTRACT: The substrate specificity of the steroid sulfate-hydroxylating activity in microsomes from human fetal liver has been investigated. Twelve different C_{18}, C_{19}, C_{21}, and C_{27} steroid sulfates and the corresponding free steroids were used as substrates. The introduction of a sulfate group on the steroid substrate was found to have two principal effects. (1) The hydrophilic sulfate group directs the steroid molecule so that it only interacts with the active site of cytochrome P-450 with its non-sulfurylated, hydrophobic end. (2) The sulfate group interacts with the enzyme surface resulting in exposure of a slightly different part of the hydrophobic end of the substrate to the active site of cytochrome P-450 than when the same end of the free steroid is exposed to the active site of the enzyme. As a consequence of these two effects of the sulfate group, the "steroid sulfate pathway" of steroid hydroxylations generally differs considerably from the "free steroid pathway," both from a qualitative and a quantitative aspect. This difference was found to be most pronounced with estrogens: whereas estradiol was not hydroxylated by human fetal liver microsomal preparations, estradiol 3-sulfate was both 15α- and 16α-hydroxylated. Thus, for certain steroids, sulfurylation is a prerequisite for further metabolism by microsomal hydroxylase systems. These results indicate the presence in human fetal liver microsomes of a multipotent, highly unspecific, hydrophobic "bulk" of cytochrome P-450. The existence of this hydroxylase system which efficiently hydroxylates steroid sulfates is probably of great physiological importance as a detoxifying mechanism in the human fetus.

The human fetal tissues have a high sulfurylase but very low sulfatase activity (Diczfalusy, 1969). Consequently, sulfoconjugated steroid hormones are present in high concentrations in fetal tissues (Huhtaniemi *et al.*, 1970). During pregnancy, steroid sulfates are known to be important precursors of other steroid hormones (Diczfalusy, 1969) and it has been suggested that the sulfoconjugated steroids may be directly metabolized without prior cleavage of the sulfate group (Huhtaniemi, 1974).

In view of these considerations and of our recent finding of a specific steroid sulfate-hydroxylating enzyme system in female rat liver (Gustafsson and Ingelman-Sundberg, 1974), we have undertaken a study of the substrate specificity of the human fetal liver microsomal enzyme system that catalyzes the hydroxylation of steroid sulfates. The results presented in this paper indicate the existence of a steroid sulfate-hydroxylating species of cytochrome P-450 in human fetal liver which is completely different from the corresponding species in rat liver.

† From the Department of Germfree Research and Department of Chemistry, Karolinska Institutet and Department of Clinical Pharmacology, Huddinge Hospital, Stockholm, Sweden. *Received August 16, 1974.* This work was supported by grants from the Swedish Medical Research Council (13X-2819 and 04X-4496), from the World Health Organization, from Expressen's Prenatal Research Fund, and from the Association of the Swedish Pharmaceutical Industry.

Materials and Methods

Steroids. Radioactive and nonradioactive steroids were synthesized or obtained as described in Table I. The sulfate conjugates of the steroids were synthesized essentially according to Mumma *et al.* (1969), purified as described before (Gustafsson and Ingelman-Sundberg, 1974), and recrystallized from acetone or methanol. Phenolic sulfate esters were liable to hydrolyze spontaneously and were incubated immediately after recrystallization. Reference steroids utilized in the identifications were obtained from sources specified previously (Berg and Gustafsson, 1973; Einarsson *et al.*, 1973a,c).

Biological Material. Human fetuses (see Table II) (22) were obtained at legal abortion for socio-medical reasons. The abortions were performed *via* hysterotomy. Only fetuses from healthy women were included in the investigation. The fetuses were immediately transported to the laboratory and preparation of the microsomal fraction of the liver was started within 45 min after abortion.

Experimental Conditions. Liver homogenates, 20% (w/v), were prepared either in a modified Bucher medium (Bergström and Gloor, 1955), pH 7.4, or in 0.25 M sucrose, with a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 9000 *g* for 10 min and the resulting supernatant was centrifuged at 105,000*g* for 70 min. The protein concentration of the microsomal fraction was determined