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^{13}C NMR STUDIES ON BIOLOGICAL SYSTEMS.

I. CARCINOGENIC POLYNUCLEAR AROMATIC HYDROCARBONS.

II. PHENACETIN METABOLISM.

• RICHARD S. OZUBKO

A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Doctor of Philosophy

Carleton University

August 1979

The undersigned recommend to the Faculty of Graduate
Studies and Research acceptance of the thesis

"¹³C NMR STUDIES ON BIOLOGICAL SYSTEMS.

I. CARCINOGENIC POLYNUCLEAR AROMATIC HYDROCARBONS.

II. PHENACETIN METABOLISM."

submitted by Richard S. Ozubko, B.Sc., in partial fulfilment
for the degree of Doctor of Philosophy.

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ABSTRACT

The ^{13}C NMR chemical shift values have been assigned for a number of carcinogenic polynuclear aromatic hydrocarbons. The compounds range from the simple two ring system, naphthalene, to ones having six condensed benzenoid rings. Also included are simple, but carcinogenically potent, methylated derivatives of some of these compounds. The reasoning for each assignment is presented in detail. Due to the narrow range of the chemical shift values it was necessary to utilize a number of assignment techniques, the most common of which was model compound chemical shifts. Single frequency selective proton decoupling and deuteration were valuable in assigning with certainty resonances of a select few carbons. The unambiguous assignment of the quaternary carbon resonances was more difficult. For those few compounds with deuterated analogues, small deuterium isotope shifts assigned those quaternary carbons near the site of deuteration. A new technique developed was based on the spin-lattice relaxation times, T_1 , of the quaternary carbons. This permitted one to distinguish those quaternary carbons as having either none, one or two adjacent protonated carbons. Due to the difficulty with measuring these long T_1 's, a case was developed for using the resonance intensity of the carbon as a measure of its relative

T₁ and proceeding from there to the molecular information. In all cases, the chemical shift assignments made by these techniques verified those made using model compounds. The approach and model compound system detailed here will be useful in assigning other polynuclear aromatic compounds. One interesting chemical shift effect noted in angularly condensed compounds is a large downfield δ shift which arises due to extreme steric crowding in these rigid systems. A brief discussion is presented regarding the ^{13}C chemical shifts and the search for an intrinsic molecular property which would relate to carcinogenic potency.

In the second part, ^{13}C NMR has been used to investigate the mechanism by which the pharmaceutical phenacetin undergoes oxidative deethylation in rat liver microsomes. Other studies had indirectly indicated the formation of a short-lived hemiacetal species as an intermediate metabolite, and another test implicated this form as being involved with the known toxicity of phenacetin. The in vitro ^{13}C NMR experiments were carried out using phenacetin ^{13}C -labelled at its methylene carbon, the site where the hemiacetal will form. The metabolism was performed with the NADPH-dependent cytochrome P-450 mixed function oxidase system of rat liver microsomes. The details of each component of the in vitro system are presented. The results

from the first experiments are closely examined and the equilibria involved in the functioning metabolic system in the NMR tube are described. These led to the method of adding acetaldehyde to the in vitro reaction mixture to alter the equilibria to favor the hemiacetal intermediate. The ^{13}C NMR evidence for the existence of the hemiacetal is presented. The approach used in this problem is applicable to investigation of the mechanism of metabolism of a number of other pharmaceuticals for which various intermediates have been proposed.

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PART I

THE ^{13}C NMR CHEMICAL SHIFTS OF
POLYNUCLEAR AROMATIC HYDROCARBONS

Chapter 1

THEORY OF THE CHEMICAL CARCINOGENESIS OF POLYNUCLEAR AROMATIC HYDROCARBONS

A. Cancer and Polynuclear Aromatic Hydrocarbons

In these times, the term "cancer" is very broad in scope and is applied to a large group of multicellular diseases which are caused by many different agents and which involve several biochemical processes. General characteristics are: the growth of malignant cells which proliferate in an apparently uncontrolled manner (in contrast to mitosis), the invasion of adjacent tissue, and the dissemination to distant tissue to form tumors. The end result is a disruption of the normal complex, well co-ordinated life process and the establishment of a parasitic abnormal tissue which can exist independent from its original stimula.

There are three general classes of agents which are known to cause this neoplastic disease (one associated with the growth of new abnormal body tissue): biological (viral in nature), physical (e.g. radiation) and chemical.

The most widely identified and documented of these are the chemical agents. These substances have a recorded history dating back to 1775. The first documented correlation between cancer and a chemical was made by Percival Pott

when he noted the cancerous lesions on the soot contaminated skin of the scrotum of chimney sweeps (1). Among the constituents of soot, the polycyclic or polynuclear aromatic hydrocarbons would later be identified as major components. Further research has found this group of carcinogens (or cancer-inducing compounds) to be constituents of coal-tars, pitches, tobacco smoke and pollutants. Despite the high chemical stability and relative lack of reactivity of the polynuclear aromatic hydrocarbons, they are among the most potent and significant classes of chemical carcinogens.

The polynuclear aromatic hydrocarbons have received much attention because of their carcinogenicity and because of the diversity with which they appear in nature (2). However, the mode of action of these compounds has remained largely unsolved. Much research is carried out because of this imprecise knowledge of the molecular level reactions involved in initiation and development of the subsequent neoplastic tissue. One very important research approach has been that of attempting to identify the ultimate carcinogen of each compound. Before any of the polynuclear aromatic hydrocarbons can exert a carcinogenic effect they must be chemically changed to an active form via metabolism. A second important aspect of the research has been the study of the derivatives of these compounds, e.g. methylated or hydroxylated, and how modification of the parent compound

at various positions affects its carcinogenic power.

There are numerous reviews and books(3,4) which have been written on these research efforts.

Another very important area of research on polynuclear aromatic hydrocarbons and one which has arisen from theoretical studies of these compounds, has been the hypotheses of their mode of carcinogenic action. Quantum mechanics has played a substantial role in the study of these "bare" molecules (i.e., away from any consideration of its interactions with biological material, be it proteins, tissue, etc.). Theoreticians have formulated hypotheses, using the intrinsic electronic properties of the molecules to explain chemical carcinogenesis and predict the reactivity of new compounds of the same family. Of these, the most successful of the theories is the K-L theory which attempts to correlate bond reactivity with carcinogenesis. It is this theory which prompted the ^{13}C NMR study of the polynuclear aromatic hydrocarbons, the main subject of this thesis. This chapter is used to summarize briefly the K-L theory of chemical carcinogenesis and to define the scope of this part of the thesis.

B. Alternant and Non-alternant Aromatic Hydrocarbons

Conjugated hydrocarbons can be defined simply as hydrocarbons for which Kekulé structures can be drawn.

There are two broad categories into which conjugated hydrocarbons can be divided: (i) the purely conjugated ones, in which the alternating single and double bonds extend over all the carbons, and (ii) the substituted conjugated ones, in which the alternating bonds do not extend over all the carbon atoms. For the purely conjugated hydrocarbons, there is a further division to alternant and nonalternant hydrocarbons. The definition of alternant is that all rings have an even number of centers in the structural figure of the molecule. If one ring has an uneven number of centers the molecule is a nonalternant one. Figure 1 gives examples for the four subgroups of conjugated hydrocarbons.

The K-L theory was only strictly developed for the pure conjugated alternant aromatic hydrocarbons. This class of compounds was attractive and amenable to such studies for a number of reasons: the whole series can be easily visualized and constructed with the very basic building block of the six-membered aromatic benzene-like ring; there is a diversity of carcinogenic properties through the series; and they occur abundantly in nature. There have been some extensions, mostly by intuitive reasoning, to simply substituted alternant polynuclear aromatic hydrocarbons (e.g. monomethyl

Figure 1. The different classes of polynuclear aromatic hydrocarbons.

PURELY CONJUGATED

Alternant Hydrocarbon

Nonalternant Hydrocarbon

SUBSTITUTED CONJUGATED

substituents at various positions). However, the theory which will now be presented deals only with the pure alternant series of compounds.

C. K-L Theory of the Carcinogenicity of Alternant Polynuclear Aromatic Hydrocarbons

The K-L theory of the carcinogenic properties of polynuclear aromatic hydrocarbons evolved from the work of several different groups. The most popular and research-provoking version emerged in 1954 and is credited to A. Pullman. There have been several minor modifications to it, but it still is essentially the same as originally stated.

The origin of the theory is the observed reactivities and sites of reactivities of the alternant aromatic compounds, especially with regards to addition reactions since it was thought these initiated carcinogenesis. One of the first complete studies involved the addition of osmium tetroxide, OsO_4 , to a series of typical alternant hydrocarbons (5). In these experiments it was observed that a meso-phenanthrenic region (like the C-9,C-10 bond in phenanthrene) was

particularly liable to be involved in addition reactions:

This region became known as the K-region and it was postulated

that such a region rich in π -electrons would be especially prone to addition reactions and a critical structural feature necessary for carcinogenic activity. With this observation two groups of French theoretical chemists, headed by the Daudels and Pullmans, attempted to correlate carcinogenic activity with various electronic properties of this K-region. Using molecular orbital theory they calculated mobile bond indexes, free valence bond indexes, "total charge" and various other electronic properties of the bonds and atoms of the K-region*. However, correlation with biological activity over the series of compounds was always poor.

It was Kooyman and Heringa who noticed that the highest free valence index rarely coincided with the atoms of the K-region. Thus, they began a study of the reactivity of the alternant hydrocarbons towards free radical substitution reactions (7), and from this a new region was defined for these compounds. This was the meso-anthracenic region.

* The reader is referred to (3), (4) and (6) for a complete discussion and definition of these terms and attempted correlations. It is sufficient for this current discussion to understand that the mobile bond order is intuitively related to the "quantity" of electrons isolated at a bond, making that bond susceptible to an addition reaction, and that the free valence index is related to the amount of excess charge at a particular atom, making it susceptible to a substitution reaction involving free radicals or electrophilic or nucleophilic reagents. The larger the mobile bond index or the free valence index the more susceptible to an addition or substitution reaction, respectively.

(like C-9,10 in anthracene) or the L-region.

Thus, two well defined and chemically distinct regions were defined for the alternant hydrocarbons. This is illustrated for benz[a]anthracene. The meso-phenanthrenic

or K-region has a high mobile bond order and was considered susceptible to addition reactions and thus an ultimate carcinogenic fate. The meso-anthracenic or L-region indicated positions of high free valence indexes which were likely to partake in substitution reactions (which were believed to lead to a noncarcinogenic fate). These definitions led directly to the K-L theory of carcinogenesis: for an alternant aromatic hydrocarbon to be carcinogenic it must possess an active K-region and if it also has an L-region this must be rather unreactive (to prevent the molecule from being drawn into reactions not leading to carcinogenesis).

A. Pullman first proposed this (8) and developed a new set of dynamic electronic indexes to put it on a quantitative basis (9). Central to the calculation was the premise that when two molecules react, the lower the energy

needed to bring them to the activated state the greater the ease by which the reaction will occur. Pullman's theory allowed for a calculation of the activation energy for the different regions of the unsubstituted polycyclic aromatic hydrocarbons. With these it was possible to assess the ease with which the different reactions would proceed.

The basis of the calculations is the concept of localization energies, the energy necessary for two π -electrons to become localized at or between certain carbon atoms so that new bonds may form. These localization energies are the difference then between the π -energy of the resonance hybrid of the polycyclic hydrocarbon (lowest energy equilibrium state) and the π -energy of the remainder of the conjugated system after a pair of π -electrons is "frozen" or localized at certain carbons (this residual conjugated portion will have less resonance energy, i.e. higher energy overall). For the alternant polynuclear aromatic hydrocarbons, three types of π -electron localization are envisioned:

(i) carbon localization

For a substitution reaction to occur by electrophilic, free radical or nucleophilic reagents, it has been hypothesized and substantiated that carbon localization is necessary and that the energy involved is independent of the number of electrons localized (two, one or none,

respectively for the above three types of substitution). Thus, the carbon localization energy, CLE, will depend only on the position of the carbon, and further, the same carbon should display the same relative reactivity to each type of attacking reagent. Therefore, the CLE of a carbon is defined as "the difference in resonance energy between the initial unpolarized hydrocarbon and the remainder of the conjugated segment after localization"(4).

(ii) bond localization

The bond localization energy, BLE, is defined as the energy necessary to localize a pair of π -electrons between two adjacent carbon atoms. This bond does not participate in conjugation, and thus the BLE in the above illustration is calculated as the difference in resonance energy between benz[a]anthracene and the 2-phenylnaphthalene moiety.

(iii) para localization of electrons

The para localization of electrons energy (PLEE) is defined as energy needed to localize two electrons simultaneously, one at each of two carbons which are para to one another. In the structure above, the PLEE would be the difference in the resonance energies between the benz[a]anthracene and the 9,10-dihydro-derivative (composed of separate naphthalenic and benzenic resonance systems).

Using these three definitions, Pullman defined two complex indices of reactivity:

- (i) ortholocalization energy, OLE; defined as the sum of BLE and the minimum of the two CLE for the two carbons involved in the bond localization of the energies
- (ii) paralocalization energy PLE; defined as the sum of the PLEE and the minimum CLE of the two para carbons.

Using either linear combination of atomic orbitals (LCAO) or valence bond (VB) methods, all the necessary energies may be calculated. The values generally come from LCAO calculations and are in units of the Huckel resonance integral β . The OLE and PLE for a number of alternant aromatic hydrocarbons, along with an index of their carcinogenic power, are given in Table 1. The most reactive bonds and para positions are indicated on each molecular structure.

From such calculations for many aromatic hydrocarbons (9), Pullman empirically derived the two elements

Table 1. Localization energies and carcinogenic potency of some common polynuclear aromatic hydrocarbons.

Hydrocarbon	Ortholocal- ization Energy (β)	Paralocal- ization Energy (β)	Carcino- genicity
(4.07)	(6.54)	-
(3.53)	5.38	-
3.36		-
3.29		0/+
3.51	5.67	0/+
3.30	5.69	++
3.23		++++

— denotes most reactive bond
 ● denotes most reactive para posn.

which constitute the K-L theory of carcinogenicity. These are:

- (i) For a polynuclear aromatic hydrocarbon to display carcinogenic activity it must possess a reactive K-region, the OLE of which should be less than or equal to 3.31 β .
- (ii) If the compound has an L-region it must be relatively unreactive so that its PLE is greater than or equal to 5.66 β .

There have been many equivalent predictions, modifications and extensions to this original formulation (4). All of these have sought to improve the correlation between the predicted and the observed carcinogenicities. Using any of these theories, one runs into exceptions if one tries to apply the rules to too wide a range of compounds. When they are applied to a limited homogeneous series, such as the alternant polynuclear aromatic hydrocarbons, the correlation is not perfect but good. Difficulties can arise even with some of these compounds since not all have a K and/or L region as they have been defined here. The theory has been highly criticized because of such difficulties and because some consider it impossible to identify a unique parameter or chemical criteria to distinguish carcinogen from non-carcinogen when such a complex multitissue process is involved. Those who support the theory have countered these criticisms with arguments based on extending some of the definitions, giving rise to terms such as "pseudo-K"

regions. Another problem for the correlation is how one arrives at a compound's carcinogenic potency, a value very dependent on such factors as method and location of application, test animal, etc.

Historically, the K-L theory was accepted by many, despite its short-comings, and was cited as proof that one should be able to elucidate one structural feature identifiable with activity. Thus, the proposal of the K-L theory led to much further research for a parameter(s) which would correlate even more closely with the carcinogenic activity. One physical approach which was utilized for such research was that of nuclear magnetic resonance spectroscopy (NMR).

D. ¹H NMR Studies of Polynuclear Aromatic Hydrocarbons

In the K-L theory for polynuclear aromatic hydrocarbons one of the important underlying principles of the implied reactivities is the behavior of the π -electron clouds. One of the ways used to obtain information about the nature of these π -electrons was the high resolution hydrogen (¹H) NMR solution spectra of these compounds. When the aromatic compounds are placed in the magnetic field of the instrument, the π -electrons circulate about the rings, a manifestation of diamagnetic anisotropy. For the hydrogens directly attached to aromatic carbons these ring currents contribute greatly

to deshielding the hydrogens from the magnetic field and thus lead to the observed ^1H chemical shifts. Since the chemical shifts are also sensitive to the local environment of the nuclei it was hoped that they would be able to provide information on the local electron density, and especially those of most interest at the K and L regions.

Some ^1H NMR studies have been undertaken to obtain information on the ring currents present in the polynuclear aromatic hydrocarbons. Using a magnetic-field modified LCAO method, the ring currents of these compounds can be calculated from the chemical shift data (10-12). The ring currents calculated are consistent with accepted views of them. For example, the two molecules below, perylene and dibenzo[cd,lm]-peryene, have very different ring currents in the central ring, the values being 0.239 and 1.446

respectively. In all the Kekulé structures which can be devised for the perylene molecule, the central bonds which join the two naphthalenic units always appear as single bonds. For dibenzo[cd,lm]perylene, Kekulé structures can be drawn so that all the bonds of the central ring can participate in the conjugation. Thus, the calculated ring currents of the central rings of the two compounds reflect

the expected absence and presence of such. It was noted that the dibenzo[cd,lm]perylene is an active carcinogen (one of the few heptacyclic or higher polynuclears to be so) while perylene is inactive. There have been ^1H NMR studies on most of the alternant polynuclear aromatics (11-14), but a correlation between the ring currents and carcinogenic activity has not been found.

Complete ^1H NMR chemical shifts assignments have been obtained (13-17) for most of the polynuclear aromatic hydrocarbons and some of their derivatives which are important carcinogens (such as 7,12-dimethylbenz[a]anthracene and 3-methylcholanthrene). Again, no correlation has ever been found between the chemical shifts of the protons attached to the molecule or even those at K or L regions and the carcinogenic potency. For sterically hindered regions of the polycyclics, such as for the protons in a bay region (illustrated), the ^1H chemical shifts are subjected to large

BAY REGION

downfield chemical shifts and are thus sensitive to the non-planar geometry of the region. In derivatives where a substituent is placed in the bay region, such as in 7,12-dimethylbenz[a]anthracene, the chemical shifts of the protons have been used as an indication of a new

stereochemistry in various regions of the molecule, including the K and bay regions. Thus, there has been a suggestion that the carcinogenic activity is associated with the stereochemistry of the substituent in the L-region and with a geometric factor in the K-region (17).

It was noted by one group that proton coupling constants often have a linear relationship with the π -bond order of the intervening bonds as calculated using Huckel molecular orbital theory (18). Due to a proposed relationship between π -bond order and bond length, it was suggested that the high resolution ^1H NMR ortho coupling constants should correlate with bond length, bond order and thus carcinogenic activity. While small planar polynuclear aromatics follow this, a study by Bartle, Jones and Matthews showed that for a more complete series of the alternant hydrocarbons there was no such relationship between the K-regions's ortho coupling constant, π -bond order and carcinogenic activity (19). The K-regions of various carcinogens with similar potency all showed relatively large differences in these coupling constants and π -bond orders (as calculated from them).

For the polynuclear aromatic hydrocarbons there have been few ^1H NMR studies other than those mentioned above. The main goal of these efforts has been to correctly assign the ^1H chemical shifts and coupling constants, a very formidable task for the larger unsymmetric molecules. In the few papers where some ^1H NMR parameter is compared to carcinogenic activity, no observable correlation has yet

been found.

E. ^{13}C NMR for Studying the Polynuclear Aromatic Hydrocarbons.

Scope of the Thesis Project.

The use of ^1H NMR to study this series of compounds is complicated by a number of serious limitations inherent with the technique. As will now be detailed, it is the absence of these complications in ^{13}C NMR that makes it desirable to undertake such studies.

The first and most obvious problem of ^1H NMR is that it is not gathering information directly at the centers of interest, the carbon atoms. One desires to understand the reactivity of the carbon atoms but with ^1H NMR one can only go about this by studying their directly bonded hydrogen atoms. However, not all carbons have directly bonded hydrogens. ^1H NMR cannot provide information for the ring junction or substituted carbons in an aromatic molecule. The use of ^{13}C NMR allows direct observation of the carbon at each position of the molecular backbone.

The second problem with the use of ^1H NMR is that the spectra are very complicated due to the presence of ^1H - ^1H spin-spin coupling. This results in multiplets appearing for most every hydrogen in a typical polynuclear aromatic hydrocarbon. Figure 2 shows the aromatic regions of the ^1H and ^{13}C NMR spectra of benz[a]anthracene. As is evident, the ^1H NMR spectrum is very complex even at the high field

Figure 2. The ^1H and ^{13}C NMR spectra of benz[a]anthracene.

A. 220 MHz ^1H NMR spectrum of benz[a]anthracene in CS_2 (15).

B. 25 MHz proton decoupled ^{13}C NMR spectrum of benz[a]anthracene in CDCl_3 .

at which it was obtained (the Larmor frequency for ^{13}C at the same field would be 55.4 MHz). Complete analysis for the ^1H chemical shifts and coupling constants would require computer simulation. In contrast, the proton decoupled ^{13}C spectrum has only one line per carbon atom and these can be much more easily assigned. In addition, this same feature allows one to observe readily small localized modifications, e.g. methyl substitution, of the molecule. For the same change, the ^1H NMR spectrum may obscure detail in its complex multiplet nature, and one is confronted with having to analyze by computer a new complex spectrum even for such a minor chemical modification.

The third problem with ^1H NMR investigations is that the chemical shift values are not as sensitive to structural effects as those of ^{13}C NMR. A chemical modification to a molecule is manifested very simply in ^{13}C NMR, partially due to each carbon being represented by one line in the spectrum. Further, the effect of the molecular modification on the ^{13}C chemical shifts is large and can be predicted since substituent effects, both at the site and at the adjacent carbons, are well documented in the literature (see for example (20)). The ^1H NMR spectrum characteristically displays aromatic hydrogens over 2.5 ppm or less while the ^{13}C chemical shift range for aromatic carbons is approximately 25 ppm. Thus, it is easier to "understand" the molecule using ^{13}C NMR and this is direct information

on the actual atoms making up the framework of the structure.

For these reasons it was considered desirable to obtain and analyze the ^{13}C NMR spectra of the polynuclear aromatic hydrocarbons. It was hoped that these ^{13}C chemical shifts would lead to a better understanding at a molecular level of the chemical nature of this series of carcinogenic compounds. Potentially these ^{13}C chemical shifts should contain more valuable information regarding the local electronic properties at each carbon than the ^1H chemical shifts of the hydrogen bonded to some of them. In addition to this application of the ^{13}C chemical shifts, that is with regards to the electronic nature of a carbon and its implication to carcinogenic activity, they are of interest in a more fundamental way. These compounds constitute an important basic series of aromatic compounds since many compounds of chemical or biological interest are derived from them. Thus, the ^{13}C chemical shifts are an important starting point for studies carried out on these related compounds.

In this part of the thesis, the ^{13}C NMR spectra of some of the more important alternant polynuclear aromatic hydrocarbons are presented. The logic used in making the assignments of the resonances of each compound is explained in detail. The compounds studied range in size from dicyclics to hexacyclic. Also analysed are some methylated derivatives of alternant aromatic hydrocarbons and two nonalternant aromatic hydrocarbons. These have been included because

they are important carcinogens (e.g., 7,12-dimethylbenz[a]-anthracene, 3-methylcholanthrene) and because they are based on an alternant aromatic hydrocarbon included in this current study. The experimental details for sample preparations and instrumental parameters and techniques are given in Chapter 2. The complete analysis of these spectra follows in Chapter 3. In Chapter 4 the conclusions inferred from these results are summarized and a list of suggestions for further work is given.

Chapter 2

MATERIALS AND METHODS

A. Materials

The structures and the numbering schemes (21) for the fused polynuclear hydrocarbons studied are presented in Figure 3. The systematic names based on the 1957 IUPAC Rules for Nomenclature of Organic Chemistry, and the generally accepted names (21) (where such exist or are different from the IUPAC name) are given in Table 2. While the compounds under discussion will for the most part be referred to by their compound number, the † in Table 2 indicates which name will be used for each compound when it is necessary to refer to that compound by name in the text.

Compounds 1 and 5 were purchased from Eastman Organic Chemicals (Rochester, New York), and compounds 6, 7, 8, 11, 12, 13, 14, 15, 17, 20, 21, 22, 24, 25, 26, and 27 were obtained from K and K Laboratories Inc. (Plainview, New York). Samples of 2, 9, and 10 were generous gifts of Drs. L. C. Leitch and R. Renaud (National Research Council of Canada, Ottawa, Ontario). All compounds were used without further purification. The trideuterio derivative of benz[a]pyrene (23) was synthesized by the method of Cavaliere and Calvin (22,23).

The deuterated chloroform used as a solvent contained 1% (v/v) tetramethylsilane (TMS) and was purchased

Figure 3. Structures and numbering schemes of the polynuclear aromatic hydrocarbons.

1 2 3 4 5 6 7

Figure 3. (continued)

8 9 10 11 12 13

Figure 3. (continued)

14 15 16 17 18 19 20

Figure 3. (continued)

21 22 23 24 25 26 27

Table 2. Compound number, IUPAC systematic name and common name (where different or applicable from the IUPAC name).

Compound Number	IUPAC Name	Common Name Or Abbreviation
<u>1</u>	naphthalene	†
<u>2</u>	1,4,5,8-tetradeuterionaphthalene	†
<u>3</u>	1-methylnaphthalene	†
<u>4</u>	1,4-dimethylnaphthalene	†
<u>5</u>	anthracene	†
<u>6</u>	9,10-dimethylantracene	†
<u>7</u>	9-methylantracene	†
<u>8</u>	phenanthrene	†
<u>9</u>	9,10-dideuteriophenanthrene	†
<u>10</u>	4,5-dideuteriophenanthrene	†
<u>11</u>	triphenylene	† 9,10-benzophenanthrene; isochrysene
<u>12</u>	dibenz[a,c]anthracene	† 1,2,3,4-dibenzanthracene
<u>13</u>	dibenz[a,h]anthracene	† 1,2,5,6-dibenzanthracene
<u>14</u>	benz[a]anthracene	† 1,2-benzanthracene; B[a]A; BA
<u>15</u>	7-methylbenz[a]anthracene	† 9-methyl-1,2-benzanthracene
<u>16</u>	1,8-dimethylnaphthalene	†
<u>17</u>	7,12-dimethylbenz[a]anthracene	† 9,10-dimethyl-1,2-benzanthracene; DMBA
<u>18</u>	acenaphthene	†
<u>19</u>	2-methylnaphthalene	†
<u>20</u>	3-methylcholanthrene	† 3-methylbenz[j]aceanthrylene; 20-methylcholanthrene

Table 2. (cont.)

Compound Number	IUPAC Name	Common Name Or Abbreviation
<u>21</u>	pyrene	+ benzo[def]phenanthrene
<u>22</u>	benzo[a]pyrene	+ 3,4-benzopyrene; B[a]P; BP; 1,2-benzopyrene;
<u>23</u>	1,3,6-trideuteriobenzo[a]pyrene	+
<u>24</u>	perylene	+ dibenz[de,kl]anthracene
<u>25</u>	benzo[ghi]perylene	1,12-benzoperylene +
<u>26</u>	dibenzo[def,mno]chrysene	anthanthrene +
<u>27</u>	naphtho[1,2,3,4-def]chrysene	dibenzo[a,e]pyrene; + 1,2,4,5-dibenzopyrene

† Name of compound used in text

from Merck, Sharpe and Dohme Canada (Montreal, Quebec).

B. Sample Preparation

All spectra obtained or quoted are of the compounds dissolved in CDCl_3 . In general, the larger the number of condensed rings the lower the solubility of the compound in the CDCl_3 . Alkylation causes an increase in the solubility of the same compounds. Less material was available of the larger ring systems (except for the common carcinogens such as 13, 14, 15, 17, 20, 21 and 22). The solubility problem and the amount of material available prevented the obtaining of ^{13}C NMR spectra of all compounds at the same concentration. In general, 10 to 125 mgm of the compound was placed in 1.0 ml of CDCl_3 , the sample heated or vortexed to facilitate solubilization, and the resulting solution or suspension transferred to a 12 mm NMR tube. The concentrations run are all estimated (for the lower values) in the range of 0.04 to 0.40 M -- the majority of the chemical shifts reported are for solutions ≤ 0.10 M.

In many cases, low solubility and/or very long T_1 's prevented observation of the low intensity, non-protonated ring junction carbon atoms (all carbons with a number having an a, b, or c after them). To observe these, the paramagnetic relaxation reagent tris(acetylacetonato)chromium(III), $\text{Cr}(\text{acac})_3$, was added to the solutions to a final concentration of 5×10^{-2} M. At this concentration

1
Cr(acac)₃ has been shown to be inert and to cause no changes in chemical shift. It equalizes and shortens the T₁'s (i.e. T₁ ≤ 0.5) of all carbon atoms of uncharged organic molecules, and increases the intensities of the non-protonated carbons so that they manifest approximately their correct intensities with respect to those of the protonated carbons (24-26).

When the deuterium isotope shifts were used to aid in making the assignments in the series 8, 9, and 10, and 22 and 23, equimolar concentrations (0.33 M and 0.24 M respectively) were used in each of the series in order to eliminate any dependence of the chemical shifts on the concentration. Ideally, equimolar solutions should have been used in the measurement of the spin-lattice relaxation times (T₁) of 8, 9, 10, and 12. However, due to the long T₁'s and the low resonance intensities of the non-proton bearing carbons of interest, the signal-to-noise ratio (and thus experimental time required) had to be optimized by using concentrated solutions. This led to solutions using maximum and subsequently different amounts of the compounds dissolved in a minimum volume of 1 ml of CDCl₃.

C. Spectrometer Operation and Chemical Shift Measurement

Natural abundance ¹³C nuclear magnetic resonance spectra of the compounds 1, 2, 5-15, 17 and 20-27 were obtained at 25.16 MHz (2.3487 Tesla) on a Varian XL-100-15

spectrometer operating in the pulse Fourier Transform (FT) mode. All spectra were recorded with broad-band noise-modulated proton decoupling at a probe temperature of 32°C (as periodically determined using a thermocouple). The aromatic region of each compound was obtained using a 1000 Hz spectral window and a minimum of 4096 data points. Typically, an acquisition time (equivalent to pulse repetition time since no delays were used) of 2.0 seconds and a 55° pulse flip angle (a 90° pulse angle required a 65 µsecond pulse width) were used. Where a greater resolution was required to observe closely-lying resonances 8192 data points were used. For the compounds with aliphatic carbons, a spectral width of 5000 Hz and 8192 data points were used. All free-induction decays (FID) were stored in a Varian 620/L computer (16K memory). After a sufficient number of accumulations, the FID's were zero-filled where necessary (where less than 8192 data points were used) and then smoothed with an exponential weighting function (of varying time constants) to increase the signal to noise (S/N) ratio. The 8K FID's were apodized and subsequently Fourier transformed. The heteronuclear field/frequency lock was obtained from the deuterium of the CDCl₃ solvent.

All reported chemical shifts are expressed as downfield from internal tetramethylsilane (TMS) and are obtained by a computer data reduction sub-routine utilizing the fold-back CDCl₃ triplet (δ CDCl₃ int = 77.01). Using

the minimum of 4K data points (acquisition time of 2.0 seconds in the common 1000 Hz sweep width) leads to the condition where the chemical shifts are correct to ± 0.02 ppm and lines 0.02 ppm apart are resolvable. Since the majority of the resonances fall within a very small spectral region, chemical shifts as small as 0.02 ppm are of interest; the chemical shifts in the tables are therefore reported with two significant figures after the decimal place.

The measurement of the chemical shifts differences between compounds becomes especially critical when β -deuterium isotope shifts are used in assigning resonances. This is the case for two series; 8, 9, and 10, and 22 and 23. In both these series identical conditions were used for obtaining the spectra of the equimolar solutions; 1000 Hz sweep width, 8192 data points (4.0 second acquisition time with no pulse delay), 55° pulse flip angle and the same sweep offset as verified with a digital frequency counter. In further proof of the identity of experimental conditions in these series (and the other spectra), the line positions (as measured to 0.1 Hz from the carrier frequency) of the folded CDCl_3 triplet were found to be the same.

Single frequency selective proton decoupling was carried out by proton irradiation using a low decoupler power with no noise modulation. The decoupler offset and power level were calibrated with a sample of TMS in CDCl_3 . The experimentally determined optimal conditions for use of

this technique were a power level of 104 dB with the decoupler offset for the complete-proton decoupling of TMS being 44813.8. Thus, in these experiments, the decoupler was positioned on the proton of interest by using the following equation:

$$\text{Decoupler Offset} = 44813.8 + (100 \times \delta_{1H})$$

where δ_{1H} is the chemical shift in ppm of the proton of interest

When a selective 1H irradiation led to a sharp line in a region of the ^{13}C spectrum where many resonances were very close together, a graphical method (27) was employed to determine accurately the chemical shift of the totally decoupled carbon. Thus, despite the narrow range over which many of the ^{13}C chemical shifts fell, the single frequency selective proton decoupling technique or its graphical modification made unambiguous assignments possible for those compounds having an assigned 1H NMR spectrum.

Spin-lattice relaxation times were measured using the saturation recovery technique (28). The pulse sequence employed was

$$[90^\circ, \text{HSP}, \tau, 90^\circ, \text{AT}, \text{HSP}]_n$$

where HSP is a 20 msec homogeneity spoiling pulse along the z axis of the magnetic field, and τ is a variable interval. The nuclear magnetization has a behavior which follows equation

$$M_t = M_0 [1 - \exp(-\tau/T_1)]$$

The experimental points were fitted to this equation by a

least-squares computer program which also subjected the data to a statistical analysis. The standard deviations are given in brackets immediately after each T_1 value. The spectrometer hardware modifications and the 16K software program necessary for this homospoil method for T_1 measurement were supplied by Ellis and Peters (29).

Chapter 3

ASSIGNMENT OF ^{13}C CHEMICAL SHIFTS OF POLYNUCLEAR AROMATIC HYDROCARBONS

A. Approach to the Problem

In this chapter the ^{13}C chemical shifts are presented for the polynuclear aromatic hydrocarbons and their analogues. For these compounds all of the chemical shifts fall within 27 ppm, and 95% of these occur within 12 ppm. This represents a very small part of the total ^{13}C chemical shift range (approximately 230 ppm) of naturally occurring uncharged organic molecules. The factors leading to these closely spaced but individual resonances are complicated and for the most part inseparable. At present, neither the theory of ^{13}C chemical shifts nor established empirical relationships allow one to predict accurately the ^{13}C chemical shifts of an aromatic hydrocarbon in solution.

These factors greatly complicate resonance assignments. To overcome these problems a variety of experimental techniques have been used which individually, but most often collectively, allow unambiguous assignments to be made. A summary of the techniques used to make the spectral assignments is as follows:

- (i) chemical shift comparison by approximating chemical shifts in portions of a molecule from those of simpler model compounds of a closely

related nature

- (ii) simple empirical chemical shift additivity correlations for substituents
- (iii) single frequency selective proton decoupling
- (iv) deuterium isotope labelling
- (v) spin-lattice relaxation time measurements
- (vi) intensity alterations with relaxation reagents

Of these techniques the one relied upon most heavily was the use of model compounds. The general strategy was to attempt first the assignment of as many carbons as possible using carefully chosen model compounds, and then to confirm independently such predictions with one of the more absolute techniques mentioned above. However, most of the techniques giving unambiguous assignments can only be used to assign successfully a few carbons in each molecule. Specific deuteration in the hydrocarbons is only facile at a few positions in very few compounds. Selective proton decoupling is limited by the very highly complex, second-order proton spectra, and again results in its applicability to a few carbons in the few compounds with analysed proton spectra. Spin-lattice relaxation time arguments and the use of relaxation reagents are limited to carbons with no directly attached hydrogens, and these techniques are less certain than the previously mentioned deuteration and selective decoupling methods.

Thus, the majority of assignments must be based on

the chemical shift comparison with model compounds and the use of empirical chemical shift additivity relationships from much simpler aromatic compounds. Initially the confidence in such predictions was questionable. However, the use of the techniques giving unambiguous assignments always confirmed the predicted chemical shift or indicated an adjacent resonance (with very nearly the same chemical shift) as that belonging to the carbon in question. Since there is this evidence for the validity of the model system, a guarded confidence can be expressed for the ^{13}C chemical shift assignments of those carbons which are obtained from these model compounds and which cannot be verified by an unambiguous technique.

This chapter presents the chemical shift data and outlines the available evidence from the various techniques. The data and assignment reasoning proceed from the very simple two ring naphthalene (1) molecule and progress towards the polynuclear molecules having six condensed aromatic rings. In this way the assignments are carefully obtained from chemical shifts verified by unambiguous techniques to serve as models for more complex ones.

B. ^{13}C Chemical Shifts of the Polynuclear Aromatic Hydrocarbons

The ^{13}C chemical shift assignments for compounds 1 to 27 (as shown in Figure 3) are given in Tables 3 to 11. In these tables are the results for compounds, 3, 4, 16, 18 and 19, which have not been run or studied in the present

Table 3. ^{13}C chemical shifts of the ring carbons of 1 - 4
(ppm downfield from internal TMS).

Position	Compound			
	<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>
C-1	127.92	127.58	134.0	132.1
C-2	125.84	125.70	126.4	126.0
C-3	125.84	125.70	125.3	126.0
C-4	127.92	127.58	126.2	132.1
C-4a	133.53	133.42	133.4	132.5
C-5	127.92	127.58	128.3	124.4
C-6	125.84	125.70	125.2 ₈	125.1
C-7	125.84	125.70	125.4	125.1
C-8	127.92	127.58	123.9	124.4
C-8a	133.53	133.42	132.5	132.5

- 1 - naphthalene
2 - 1,4,5,8-tetradeuterionaphthalene
3 - 1-methylnaphthalene
4 - 1,4-dimethylnaphthalene

Table 4. ^{13}C chemical shifts of the ring carbons of 5 - 7
(ppm downfield from internal TMS).

Position	Compound		
	<u>5</u>	<u>6</u>	<u>7</u>
C-1	128.34	125.29	124.66(a)
C-2	125.24	124.66	125.22(a)
C-3	125.24	124.66	124.77(a)
C-4	128.34	125.29	129.05
C-4a	131.62	129.93	131.52
C-5	128.34	125.29	129.05
C-6	125.24	124.66	124.77(a)
C-7	125.24	124.66	125.22(a)
C-8	128.34	125.29	124.66(a)
C-8a	131.62	129.93	130.18
C-9	126.11	128.29	130.02
C-9a	131.62	129.93	130.18
C-10	126.11	128.29	125.29
C-10a	131.62	129.93	131.52

(a) denotes possible interchange of assignments

5 - anthracene

6 - 9,10-dimethylantracene

7 - 9-methylantracene

Table 5. ^{13}C chemical shifts of the ring carbons of 8 - 10
(ppm downfield from internal TMS).

Position	Compound		
	<u>8</u>	<u>9</u>	<u>10</u>
C-1	128.51	128.49	128.51
C-2	126.50	126.50	126.50
C-3	126.50	126.50	126.40
C-4	122.61	122.63	122.34
C-4a	130.30	130.30	130.22
C-4b	130.30	130.30	130.22
C-5	122.61	122.63	122.34
C-6	126.50	126.50	126.40
C-7	126.50	126.50	126.50
C-8	128.51	128.49	128.51
C-8a	132.03	131.97	132.02
C-9	126.86	126.70	126.86
C-10	126.86	126.70	126.86
C-10a	132.03	131.97	132.02

8 - phenanthrene

9 - 9,10-dideuteriophenanthrene

10 - 4,5-dideuteriophenanthrene

Table 6. ^{13}C chemical shifts of the ring carbons of 11 - 13
(ppm downfield from internal TMS).

Position	Compound		
	<u>11</u>	<u>12</u>	<u>13</u>
C-1	123.20	123.41 (a)	122.83
C-2	127.11	127.57 (b)	126.91 (c)
C-3	127.11	127.44 (b)	126.73 (c)
C-4	123.20	123.66 (a)	128.57
C-4a	129.72	130.10	132.03
C-4b	129.72	130.10	
C-5	123.20	123.66 (a)	127.13 (d)
C-6	127.11	127.44 (b)	127.41 (d)
C-6a			130.77
C-7	127.11	127.57 (b)	122.11
C-7a			129.09
C-7b			130.21
C-8	123.20	123.41 (a)	122.83
C-8a	129.72	130.19	
C-8b	129.72	128.49	
C-9	123.20	122.03	126.91 (c)
C-9a		132.28	
C-10	127.11	128.09	126.73 (c)
C-11	127.11	126.04	128.57
C-11a			132.03
C-12	123.20	126.04	127.13 (d)
C-12a	129.72		
C-12b	129.72		
C-13		128.09	127.41 (d)
C-13a		132.28	130.77
C-14		122.03	122.11
C-14a		128.49	129.09
C-14b		130.19	130.21

(a), (b), (c), (d) denote possible interchange of assignments

11 - triphenylene

12 - dibenz[a,c]anthracene

13 - dibenz[a,j]anthracene

Table 7. ^{13}C chemical shifts of the ring carbons of 14 - 17
(ppm downfield from internal TMS)

Position	Compound			
	<u>14</u>	<u>15</u>	<u>16</u>	<u>17</u>
C-1	122.83	123.64	135.2	130.25
C-2	126.71(a)	126.82	129.2	126.34(c)
C-3	126.65(a)	126.82	124.8	126.41(c)
C-4	128.49	128.36	127.7	127.43
C-4a	131.89	131.68	135.4	133.19
C-5	126.95	126.94	127.7	125.50
C-6	126.95	123.09	124.8	123.77
C-6a	131.89	130.46		130.49
C-7	127.21	130.87	129.2	127.74
C-7a	131.85	129.76		129.09
C-8	128.32	124.46	135.2	125.12(d)
C-8a			132.9	
C-9	125.67(b)	125.67		124.71(e)
C-10	125.56(b)	125.24		124.86(e)
C-11	127.63	129.34		125.30(d)
C-11a	130.56	131.53		129.09
C-12	121.42	120.30		131.33
C-12a	128.79	128.64		128.98
C-12b	130.47	131.01		132.25

(a), (b), (c), (d), (e) denote possible interchange of assignments

14 - benz[a]anthracene

15 - 7-methylbenz[a]anthracene

16 - 1,8-dimethylnaphthalene

17 - 7,12-dimethylbenz[a]anthracene

Table 8. ^{13}C chemical shifts of the ring carbons of 18 - 20
(ppm downfield from internal TMS).

Position	Compound		
	<u>18</u>	<u>19</u>	<u>20</u>
C-1	30.2	126.7	29.73(a)
C-2	30.2	135.2	29.69(a)
C-2a	145.6		142.17
C-3	118.9	127.9	128.34
C-4	127.5	127.2	130.74
C-4a		131.6	
C-5	122.0	127.5	122.82
C-5a	131.4		127.43
C-6	122.0	124.8	116.32
C-6a			129.92
C-6b			131.70
C-7	127.5	125.7	123.29
C-8	118.9	127.4	126.56(b)
C-8a	145.6	133.5	
C-8b	139.0		
C-9			126.44(b)
C-10			128.65
C-10a			131.93
C-11			125.86
C-12			123.76
C-12a			125.36
C-12b			143.22
C-12c			137.75

(a), (b) denote possible interchange of assignments

18 - acenaphthene

19 - 2-methylnaphthalene

20 - 3-methylcholanthrene

Table 9. ^{13}C chemical shifts of the ring carbons of 21 - 23
(ppm downfield from internal TMS).

Position	Compound		
	<u>21</u>	<u>22</u>	<u>23</u>
C-1	125.06	124.61(a)	124.30(d)
C-2	125.96	125.78	125.67
C-3	125.06	124.68(a)	124.37(d)
C-3a	131.30	131.21(c)	131.14(f)
C-4	127.50	127.33	127.28
C-5	127.50	127.66	127.61
C-5a	131.30	129.71	129.62
C-6	125.06	125.38	125.06
C-6a		131.46	131.39
C-7	125.96	128.77	128.71
C-8	125.06	125.88(b)	125.88(e)
C-8a	131.30		
C-9	127.50	125.91(b)	125.91(e)
C-10	127.50	122.87	122.87
C-10a	131.30	127.22	127.21
C-10b	124.84	128.10	128.10
C-10c	124.84		
C-11		122.00	122.00
C-12		128.00	127.95
C-12a		131.27(c)	131.20(f)
C-12b		125.27	N.O.
C-12c		123.58	N.O.

(a), (b), (c), (d), (e), (f) denote possible interchange of assignments

N.O. - not observed

21 - pyrene

22 - benzo[a]pyrene

23 - 1,3,6-trideuteriobenzo[a]pyrene

Table 10. ^{13}C Chemical Shifts of the Ring Carbons of 24-27
(ppm downfield from internal TMS.)

Position	Compound			
	<u>24</u>	<u>25</u>	<u>26</u>	<u>27</u>
C-1	120.15	126.42	125.89 (a)	126.11
C-2	126.48	126.42	125.85 (a)	126.64
C-2a		132.17		
C-3	127.78	127.30	123.87	120.25
C-3a	134.95		N.O.	N.O.
C-3b				N.O.
C-4	127.78	127.30	128.20 (b)	123.84 (c)
C-4a		129.13		
C-4b		125.61		
C-4c		123.83		
C-5	126.48	125.53	128.39 (b)	127.55 (d)
C-5a			N.O.	
C-6	120.15	126.12	124.16	127.75 (d)
C-6a	131.24		N.O.	
C-6b	131.24			
C-7	120.15	120.60	125.89 (a)	123.66 (c)
C-7a		130.34		N.O.
C-7b		130.34		N.O.
C-8	126.48	120.60	125.85 (a)	120.51
C-8a				N.O.
C-9	127.78	126.12	123.87	129.11
C-9a	134.95		N.O.	
C-9b	128.73			
C-10	127.78	125.53	128.20 (b)	126.25
C-10a		129.13		
C-11	126.48	127.30	128.39 (b)	126.25
C-11a			N.O.	
C-11b			N.O.	
C-11c			N.O.	
C-12	120.15	127.30	124.16	122.82
C-12a	131.24	132.17	N.O.	N.O.
C-12b	131.24	123.83	N.O.	N.O.
C-12c	128.73	125.61	N.O.	
C-13				121.89
C-14				127.87
C-14a				N.O.
C-14b				N.O.
C-14c				N.O.

(a), (b), (c), (d) denote possible interchange of assignments

N.O. - not observed

24 - perylene

25 - 1,12-benzoperylene

26 - anthanthrene

27 - dibenzo[a,e]pyrene

Table 11. ^{13}C chemical shifts of the methyl carbons
(ppm downfield from internal TMS).

Position	Compound									
	<u>3</u>	<u>4</u>	<u>6</u>	<u>7</u>	<u>15</u>	<u>16</u>	<u>17</u>	<u>19</u>	<u>20</u>	
1	19.2	19.3				25.9				
2								21.6		
3									18.65	
4		19.3								
7					14.09		14.16			
8						25.9				
9			14.02	13.83						
10			14.02							
12							20.96			

3 - 1-methylnaphthalene
 4 - 1,4-dimethylnaphthalene
 5 - 9,10-dimethylnaphthalene
 7 - 9-methylnaphthalene
 15 - 7-methylbenz[a]anthracene
 16 - 1,8-dimethylnaphthalene
 17 - 7,12-dimethylbenz[a]anthracene
 19 - 2-methylnaphthalene
 20 - 3-methylcholanthrene

work but which have been published by others. These are an integral part of the model compound system which has been used to predict many of the chemical shifts, and thus they are included for completeness and clarity.

Tables 3, 4, and 5 contain the results for naphthalene and some of its methylated derivatives, anthracene and its important methylated derivatives, and phenanthrene, respectively. These compounds establish the chemical shift regions for different carbons in linearly and angularly condensed ring systems and show the effects of ring or methyl substitution. As such, these compounds represent a very important, basic part of the model system.

Table 6 contains the results for triphenylene and the dibenzanthracenes, [a,c] and [a,h], which, although similar in structure, show different carcinogenic properties. The results for benz[a]anthracene, 7-methylbenz[a]anthracene, 7,12-dimethylbenz[a]anthracene, and a relevant dimethylnaphthalene model compound are given in Table 7. In Table 8 the chemical shifts are given for two model compounds and the potent carcinogen, 3-methylcholanthrene.

The assignments for pyrene, the well-known carcinogen benz[a]pyrene, and a deuterated benz[a]pyrene which is used to unambiguously verify the chemical shift assignments are given in Table 9.

Table 10 contains the results for some highly condensed ring systems. Complete chemical shift information

and assignments are presented for perylene and 1,12-benzoperylene. The last two compounds of Table 10, anthanthrene and dibenzo[a,e]pyrene, represent the limit to which the current study was undertaken. Due to very low solubility in CDCl_3 of these last two compounds only the carbons having directly bonded hydrogens give verifiable resonances (with the use of $\text{Cr}(\text{acac})_3$). These compounds are included to demonstrate the applicability of model systems to assign the resonances of larger and more complicated compounds.

The contents of Table 11 are the chemical shifts of all the substituent methyl groups which occur in some of the model compounds or the aromatics studied.

In most cases the assignments of the chemical shifts are unambiguous within the limits of the techniques employed. However there still exist groups of resonances which are so nearly identical in position that it is impossible to make a specific assignment. To identify members of such groups in the tables, a different lower case letter has been assigned to each group and is placed beside each group member. Despite these ambiguities, the assignments of the chemical shifts to the individual carbons is believed to be correct.

On first approaching an unassigned spectrum, the initial step was to distinguish the resonances of carbons having a directly-bonded hydrogen from those without, the so-called quaternary carbons. These quaternary carbons have a greatly reduced intensity and are easily separated

from carbons having a proton. This reduced intensity is due to two factors:

- (i) a diminished nuclear Overhauser enhancement (NOE). The NOE originates from a redistribution of spin populations of the ^{13}C energy levels brought about by the saturation of the proton transitions that takes place under the condition of continuous proton noise decoupling. When the spin-lattice relaxation takes place by the ^{13}C - ^1H dipole-dipole mechanism, this non-Boltzmann energy level distribution has been shown to lead to a theoretical signal enhancement of 2.988. For organic molecules such as those studied here, the predominant relaxation mechanism for proton-bearing carbons is this ^{13}C - ^1H dipole-dipole one and thus a full NOE is observed for them. The non-protonated quaternary carbon atoms will not necessarily receive a full NOE since other mechanisms may be involved in their relaxation. This leads to a decreased intensity relative to the protonated carbons. However, it has been shown that for molecules of a similar size, some quaternary carbons do receive a full NOE while others do not (30). The implications of this study are twofold. First, ^{13}C - ^1H dipolar relaxation is the predominant mechanism for the carbons in the molecules studied. Second, only a slight intensity decrease can be expected for the quaternary carbons due to loss of NOE, and consequently another more important

factor must be causing the greatly reduced intensities of these carbons.

- (ii) very long spin-lattice relaxation times (T_1) of the quaternary carbons. The predominant T_1 relaxation mechanism for the carbons of these aromatic hydrocarbons will be ^{13}C - ^1H dipole-dipole interaction. The expression for the ^{13}C - ^1H dipole-dipole relaxation rate, $1/T_1^{\text{DD}}$, in the extreme narrowing limit is (31):

$$\frac{1}{T_1^{\text{DD}}} = n_{\text{H}} \hbar^2 \gamma_{\text{C}}^2 \gamma_{\text{H}}^2 r_{\text{CH}}^{-6} \tau_{\text{C}}$$

where n is the number of protons involved in the dipolar relaxation at a ^{13}C - ^1H internuclear separation distance of r_{CH} . One assumes that there is a unique τ_{C} for all carbons; not a bad assumption for polynuclear aromatic hydrocarbons unless there is molecular anisotropic rotation. The consequence of the above equation for resonance intensity is better realized by looking at the C-H distances (32) involved in even the simplest condensed ring system, naphthalene. The protonated,

carbon to hydrogen distance is 1.084 Å, while the quaternary carbon's nearest hydrogen is twice that distance at 2.152 Å. Due to the inverse sixth power

relationship between r_{CH} and the dipolar relaxation rate, $1/T_1^{DD}$, the relaxation times for protonated and quaternary carbons will be very different. For naphthalene, one can estimate the ratio of the T_1 's to be:

Protonated Carbon (PC)	Quaternary Carbon (QC)
$r_{CH} = r_1$	$r_{CH} \approx 2r_1$
$n = 1$	$n = 2$
$1/T_1^{DD}_{PC} = \hbar^2 \gamma_C^2 \gamma_H^2 T_C r_1^{-6}$	$1/T_1^{DD}_{QC} = 2\hbar^2 \gamma_C^2 \gamma_H^2 T_C (2r_1)^{-6}$
$T_1^{DD}_{QC} \approx (2^5) \times (T_1^{DD}_{PC})$	
or $T_1^{DD}_{QC} \approx 32 \times T_1^{DD}_{PC}$	

While this is only an approximation to the relationship of the two T_1 values, it is verified in that all the protonated carbons of benz[a]anthracene (14) have experimental T_1 's in the range 1.8 to 2.2 s, and the quaternary carbons of phenanthrene (8) have T_1 's of 50 to 60 seconds (vide infra). It is known that the relative signal intensities in a pulse Fourier experiment are dependent on the pulse width expressed as an angle α , and on t_p/T_1 where t_p is the pulse repetition time (comprised of the acquisition time and any delay time between pulses). Figure 4 illustrates the relationship: in Figure 4.A the pulse width for maximum signal intensity is given as a function of t_p/T_1 ; in Figure 4.B the fractional signal intensity is shown as a function of t_p/T_1 for seven different pulse widths (33). With such a difference

Figure 4. The relationship between pulse width, T_1 and pulse repetition rate, t_p .

A. Pulse angle for maximum signal intensity with varying T_1 and t_p .

B. Fractional signal intensity observed for different pulse angles with varying T_1 and t_p .

in the T_1 values of the protonated and quaternary carbons of the aromatic hydrocarbons, Figure 4 says that the signal intensity of the two types of carbon will be quite different at a given pulse width and t_p . With the pulse width of 55° and the pulse repetition time of 2s, used in the current studies, one would expect a full intensity resonance for a protonated carbon and one 60% less intense for a quaternary carbon.

These two factors, along with the experimental conditions utilized, give a signal intensity for quaternary carbons with two adjacent protonated carbons of about 20% of that of the protonated carbons. For carbons with one adjacent protonated carbon, there is a further reduction, and for quaternary carbons even further removed from the protonated carbons (such as internal in a polycondensed ring system), the intensity is very low.

As an example of this, Figure 5 shows the aromatic region of benz[a]pyrene (22). The resonances of the three types of quaternary carbons and of the protonated carbons are indicated. While the distinction amongst the three types of quaternary carbons is fine, there is no difficulty in telling the protonated carbons from the quaternary carbons. This is very important in the spectral analysis and assignment because of the small chemical shift range and the limited solubility of these compounds. The other method of distinguishing such carbons, the off-resonance decoupling technique, would

Figure 5. ^{13}C NMR spectrum of benzo[a]pyrene showing the different types of protonated and quaternary carbons.

be very difficult to utilize due to the complexity which would arise in this narrow region.

This means of distinguishing protonated and quaternary carbons was always followed on first obtaining a spectrum. The other techniques previously mentioned were then used to complete the assignments.

C. Assignment of the ^{13}C Chemical Shifts of Naphthalene and its Methylated Derivatives, 1 - 4. The Results for Table 3.

Beyond benzene (which gives a simple one line spectrum) the first, most basic aromatic condensed ring system is that of naphthalene (1). The correct assignment is very important since the effects and chemical shift trends noted among naphthalene, anthracene and their methylated derivatives, and phenanthrene, are commonly invoked in the polycondensed systems to aid in making assignments.

The resonance of the quaternary carbons of naphthalene is easily assigned by its very low intensity to $\delta = 133.53$. The spectrum obtained in CDCl_3 is very similar to that reported for a CS_2 solution (34). On the basis of these original assignments, C-1,4,5,8 and C-2,3,6,7 of 1 are assigned to $\delta = 127.92$ and $\delta = 125.84$, respectively.

Due to the central role these chemical shifts must play, the assignments were unambiguously verified by the spectrum of 1,4,5,8-tetradeuterionaphthalene (2). As expected

the resonance at $\delta = 127.92$ collapsed and shifted upfield to a low intensity triplet centered at $\delta = 127.58$. The observed C-D coupling constant is $J_{C-D} = 24.5$ Hz, which is the same as that which would be predicted knowing that $J_{C-H} \approx 159$ Hz in aromatic systems and $J_{C-D} \approx J_{C-H}/6.5$. The C-2,3,6,7 and C-4a,8a resonances of 2 are shifted upfield by 0.14 ppm and 0.11 ppm, respectively.

It is interesting to note that while the C-2,3,6,7 resonance of 2 has the same intensity as 1, the C4a,8a resonance of 2 is much decreased from that observed in 1. This is a direct observation of the previously explained effect of T_1 on the signal intensity (see section B). The loss of the protons from C-1,4,5,8 has led to an appreciable increase of the T_1 of C-4a,8a due to the removal of the protons primarily responsible for their dipole-dipole relaxation in 1. Since the spectra of both 1 and 2 were run under the same conditions, the C-4a,8a resonance is observed to undergo the predicted intensity reduction.

The ^{13}C chemical shifts for 1-methylnaphthalene (3) and 1,4-dimethylnaphthalene (4) are taken from the work of Wilson and Stothers (35). Their results for these compounds differ somewhat from the original work of Doddrell and Wells (36). The former have been cited here since the ^{13}C assignments were made by the unambiguous single frequency selective decoupling technique after analysis and simulation of the proton NMR spectra. Furthermore, the former results were obtained from dilute CDCl_3 solutions (10-15% w/v) while the

original work used saturated CDCl_3 solutions or neat liquids. Comparisons with the results obtained from the dilute solutions of the current work can thus be made with greater confidence.

D. Assignment of the ^{13}C Chemical Shifts of Anthracene and its Methylated Derivatives, 5 - 7. The Results for Table 4.

The assignments for anthracene (5) in CDCl_3 have been made unambiguously. The quaternary carbon resonance is readily assignable to the peak of low intensity at $\delta = 131.62$. Of the carbons with directly-bonded hydrogens, the resonance for C-9,10 will only be half as intense as those for C-1,4,5,8 and C-2,3,6,7 and is accordingly assigned to $\delta = 126.11$. The original basis for the remaining assignments was based on the chemical shifts of C-2,3,6,7 and C-1,4,5,8 of anthracene (5) being similar to the shifts of these same carbons in naphthalene (1).

To verify these assignments of the carbons with directly-bonded hydrogens, single frequency selective proton decoupling was carried out. Using the CDCl_3 solution of 5 from the ^{13}C NMR experiment, a 100 MHz ^1H NMR spectrum showed a sharp singlet at $\delta = 8.42$ and two multiplets centered around $\delta = 7.4$ and $\delta = 8.0$. These can be assigned to H-9,10, H-2,3,6,7 and H-1,4,5,8 respectively (37). Use of these values for the selective proton decoupling confirmed the above assignments.

These results disagree with the chemical shifts

for anthracene in C_6D_6 reported by Grant and co-workers (38), unless C-9,10 undergoes an aromatic solvent-induced shift of about 6 ppm upfield. For anthracene in C_6D_6 , the correct chemical shifts were determined to be $\delta = 126.92$ and $\delta = 128.74$ for C-9,10 and C-1,4,5,8, respectively. The originally reported values of $\delta = 132.8$ and $\delta = 130.3$, respectively, were determined before the advent of the computer assisted Fourier transform method and suffered from a greatly reduced signal-to-noise ratio. The chemical shifts determined here for 5 in C_6D_6 indicate that all the carbons have aromatic solvent induced shifts of less than 1.3 ppm. The current results and assignments for anthracene in both solvents are in agreement with the more recent data of Gunther (39).

For 9,10-dimethylantracene (6) the assignments are made chiefly by use of chemical shift correlations. The two different quaternary carbon centers, C-4a,8a,9a,10a and C-9,10 are easily assigned on the basis of one low intensity quaternary line being twice the intensity of the other. The two remaining protonated carbon lines are assigned using naphthalene (1) and 1,4-dimethylnaphthalene (4) as model compounds. They predict a 3.5 ppm and a 0.7 ppm upfield shift for C-1,4,5,8 and C-2,3,6,7, respectively, of 6 relative to 5. The actual observed shifts are slightly less, being 3.05 ppm and 0.58 ppm. The upfield shift of 3.05 ppm is an example of the γ ^{13}C steric shift due to the γ relationship between C-1,4,5,8 and the methyl carbons.

The assignments for 9-methylantracene (7) can be made using intensity and chemical shift correlation arguments. The C-9 peak, $\delta = 130.02$, will be the smallest of the quaternary resonances since it arises from only one carbon. The remaining two quaternary peaks at $\delta = 131.52$ and $\delta = 130.18$ are easily assigned to C-4a,10a and C-8a,9a, respectively. This is done using C-4a and C-8a in naphthalene (1) and 1-methylnaphthalene (3), where shielding effects of 0.1 and 1.0 ppm are noted at these δ and β carbons (relative to the site of methyl substitution). Applying these shifts to anthracene, the predicted values for C-4a,10a and C-8a,9a in 7 would be $\delta = 131.5$ and $\delta = 130.6$ ppm, in good agreement with the observed ones. It would be expected that the C-8a,9a resonance should be less intense than that of C-4a,10a since these latter carbon atoms have two adjacent carbon atoms with directly-bonded hydrogens while the former, C-8a,9a, have only one such carbon. Thus, the C-4a,10a resonances will have a shorter T_1 and, under the spectral conditions, a greater intensity. This is observed in the spectrum where it is found that the peak at $\delta = 131.52$ is approximately 30% taller for the same line width.

The resonance for C-10, a proton-bearing carbon, is easily seen by inspection of peak intensities to be at $\delta = 125.29$. The remainder of the resonances are twice the area or height and belong to the four remaining sets of two carbons per set. To assign C-4,5, the 0.4 ppm downfield .

effect on C-5 noted between naphthalene (1) and 1-methylnaphthalene (3) is applied to C-4,5 in anthracene (5). The predicted shift of $\delta = 128.75$ agrees well with the only protonated carbon resonance in this region, $\delta = 129.05$. Using C-8, C-7 and C-6 respectively, in the same two model compounds, 1 and 3, the predicted chemical shifts for C-1,8, C-2,7 and C-3,6 in 7 (with those of anthracene as a basis) are $\delta = 124.3$, $\delta = 124.8$ and $\delta = 124.6$, respectively. Thus, the assignments are $\delta = 124.66$, $\delta = 125.22$ and $\delta = 124.77$ for C-1,8, C-2,7 and C-3,6 respectively, although there is some ambiguity due to the closeness of the resonances.

After these results for anthracene and its methylated anthracenes were published (40)*, Caspar et al. published the ^{13}C chemical shifts for a large series of methylated anthracenes (41). They first determined and assigned the proton chemical shifts and then used these to make the ^{13}C chemical shift assignments by the single frequency selective proton decoupling technique. The two sets of data agree for anthracene (5) and 9,10-dimethylantracene (6). The results for 9-methylantracene (7) are not in total agreement. The results from the selective decoupling experiments in (41) agree with and verify the current results for C-1,8. For C-2,7 and C-3,6, neither the selective decoupling technique nor the use of

* The results for C-2,7, C-3,6 and C-10 appear in error in reference (40).

model compounds is able to make the assignment unambiguously.

For C-10 of 7, the one protonated carbon which can easily be assigned on the basis of its intensity being one half of the other protonated carbon resonances, the results differ. Caspar et al. (41) have assigned C-10 to the same resonance as C-1,8, that most upfield. This is in contrast to the very distinctive resonance observed in the current results. This peak was most likely not seen in (41) due to a small concentration shift or an insufficient number of data points so that it could not be distinguished from $\delta = 124.8$ (equivalent to the current result of $\delta = 125.22$ in Table 4). The ^1H chemical shift of the proton on C-10 should be a distinctive singlet while all the other protons will be strongly coupled. Ordinarily this would allow for a routine selective decoupling experiment. However, the H-10 proton and the H-1,8 protons are only separated by 0.05 ppm (or 5 Hz at 100 MHz). In attempting to identify the C-10 resonance via selective decoupling, the C-1,8 resonance is also decoupled due to the proximity of H-1,8 to H-10 in the ^1H NMR. This has most likely led to the discrepancy of the results for the ^{13}C chemical shift for C-10 in 9-methylanthracene (7).

E. Assignment of the ^{13}C Chemical Shifts of the Phenanthrenes,
8 - 10. The Results for Table 5.

The correct assignment of phenanthrene (8) is of prime

importance since it is continually invoked as a model compound for all angularly condensed ring systems. This includes most of the remaining compounds for which shifts are reported.

Incomplete results for phenanthrene have been reported by three separate groups (34,42,43). Originally not all the resonances were observed (43); and when they were (34,42), the tentative assignments for C-4a,4b and C-8a,10a were in error. The assignments for all the carbons are now made unambiguously using the 9,10-dideuteriophenanthrene (9) and the 4,5-dideuteriophenanthrene (10) derivatives.

The deuterium isotope effects, which can be observed in polynuclear aromatic hydrocarbons have been studied by Martin *et al.* (44). The upfield shift effects, couplings and intensity changes for aromatic compounds can be summarized to be:

- (i) $^{13}\text{C}_\alpha\text{-D}$. The protonated carbon on which the proton is replaced by a deuterium atom undergoes a 0.27 to 0.38 ppm upfield shift. The intensity of this resonance generally undergoes a dramatic reduction under the normal conditions with which ^{13}C pulse Fourier transform spectra are obtained. This feature usually allows assignment to be made for this carbon by visual inspection of the protonated and deuterated spectra. If the concentration of the deuterated compound is sufficient, the triplet of the $^{13}\text{C}_\alpha\text{-D}$ coupling is clearly visible with a $J_{^{13}\text{C}_\alpha\text{-D}} = 24.5$ Hz.
- (ii) $^{13}\text{C}_\beta\text{-C-D}$ (ortho). The carbon β to such a substitution undergoes a 0.05 to 0.12 ppm upfield shift. When two

adjacent carbons both undergo deuteration, the effect on the β carbon can be considerably different. The observed geminal coupling is usually < 0.15 Hz as would be expected from the results for benzene (45) where $J_{^{13}\text{C}_\beta\text{-C-H}} = 1.0$ Hz and $J_{^{13}\text{C-D}} \approx J_{^{13}\text{C-H}}/6.5$.

(iii) $^{13}\text{C}_\gamma\text{-C-C-D}$ (meta). These carbons undergo no isotope shift, but line broadening due to the vicinal coupling may be observed depending on the dihedral angle.

The values of the coupling constant vary between zero and ~ 1.14 Hz.

(iv) $^{13}\text{C}_\alpha\text{-C-C-D}$ (peri*). For this, a 0.04 ppm upfield shift can be noted, arising most likely from a through space, steric mechanism. As for the meta γ effect, line broadening may be observed depending on the dihedral angle and the coupling.

By reference to these effects, the chemical shift assignments can be made unambiguously.

The chemical shifts for C-9,10 and C-4,5, $\delta = 126.86$ and $\delta = 122.61$ respectively, are easily made by inspection of the spectra of the deuterated derivatives, 9 and 10 respectively. In both, one resonance disappears and is

* The term "peri" originates from the word periphery and refers to carbons on different rings which have a relationship like the 1,8 positions in naphthalene (1).

replaced by a weak triplet which is shifted upfield 0.17 ppm in 9 and 0.27 ppm in 10. The observed $J_{1,3}C-D$ couplings are both 24 Hz. The double intensity resonance at $\delta = 126.50$ can be attributed to C-2,3,6,7 in 8. Carbons 2,7 and 3,6 should be the only carbons which will be chemically alike enough to appear at the same chemical shift. It is expected and realized that the C-3,6 resonance in 10 will be shifted upfield by about 0.1 ppm, being β to the deuterium substituted carbons at positions C-4,5. The last protonated carbons C-1,8, are assigned to $\delta = 128.51$. These are expected to have a chemical shift similar to C-1,4,5,8 of naphthalene (1) and anthracene (5). Also, this resonance is shifted upfield, as expected, by 0.02 ppm in the 9,10-deuterated phenanthrene, 9.

The deuterium isotope shifts are especially valuable for assigning the quaternary carbon centers. In the previous studies (34,42,43) there was no possibility for unambiguous assignment. However, each of the two deuterated phenanthrene derivatives has a different quaternary carbon β to a site of substitution and thus comparison of the shifts in the three phenanthrenes permits the final assignments to be made. The observed 0.06 ppm upfield shift of the $\delta = 132.03$ resonance in the 9,10-dideuteriophenanthrene (9) and the lack of any such shift (± 0.01) in 10 identifies this resonance as C-8a,10a. From the observed 0.08 ppm upfield shift of the $\delta = 130.30$ peak in 4,5-dideuteriophenanthrene (10), this resonance is

assigned to C-4a,4b. This resonance remains unshifted in 9.

As an alternative to isotope shifts, the various ^{13}C -D couplings might be used to attempt the assignment of the quaternary carbons. The basis of this is the previous observation that geminal ^{13}C -D coupling ($J_{^{13}\text{C}-\text{C}-\text{D}}$) is small (<0.15 Hz) and that vicinal coupling ($J_{^{13}\text{C}-\text{C}-\text{C}-\text{D}}$) varies between zero and >1 Hz depending upon the dihedral angle. Such an approach is not applicable to 9,10-dideuteriophenanthrene (9) because each of the quaternary carbons, C-4a, C-4b, C-8a and C-10a, is trans to one or the other deuterium atoms. Only a slight difference in vicinal couplings due to different paths might be expected. However, a rather substantial difference is found between the two coupling pathways. The resonance at $\delta = 131.97$ (assigned to C-8a,10a above) is visibly a triplet with a splitting constant of $J_{^{13}\text{C}-\text{C}-\text{C}-\text{D}} \approx 1.3$ Hz, whereas the $\delta = 130.30$ peak (attributed to C-4a,4b above) is a single line with a width at ca. 1.1 Hz (coupling ≤ 0.4 Hz). Thus, the coupling pathway $\text{D}_{10}\text{C}_{10}\text{C}_9\text{C}_{8a}$ is more effective than $\text{D}_9\text{C}_9\text{C}_{8a}\text{C}_{4b}$.

In contrast, the 4,5-dideuteriophenanthrene (10) has two different dihedral angles. For the deuterium at position 4, C-4b has a dihedral angle of ca. 0° while C-10a has one of ca. 180° . Thus, depending on the symmetry of the relationship linking the vicinal couplings and the dihedral angles, approximately equal and large (≥ 1 Hz) deuterium coupling to both C-4b and C-10a (or C-4a and C-8a) are

expected. In the spectrum of 10, the $\delta = 132.02$ resonance (attributed to C-8a, 10a above) has a width of ca. 2.5 Hz (coupling ≤ 0.8 Hz) and that of $\delta = 130.22$ (attributed to C-4a, 4b above) has a width of ca. 1.8 Hz (coupling ≤ 0.6 Hz). The fact that the trans arrangement leads to a slightly greater coupling is in agreement with that observed in ^1H - ^1H vicinal couplings (46). Accordingly, the small magnitude of the vicinal ^{13}C -D couplings and their sensitivity to the nature of path, make them an unreliable means of assignment of the quaternary carbons in these molecules.

Another method of assignment for the quaternary carbons in these phenanthrenes (8-10) utilizes spin-lattice relaxation times. Up to now, only estimates of the T_1 values --- for two resonances representing the same number of carbon atoms, the smaller the intensity the longer the T_1 , and vice versa --- have been used to verify quaternary assignments.

As previously mentioned, various types of non-protonated carbons should be distinguishable on the basis of their T_1 values, which are in turn determined by the distances to nearby protons when the predominant relaxation mechanism is dipole-dipole. For aromatic quaternary carbons one would expect an increasingly larger T_1 as one progresses from a carbon with one adjacent protonated carbon, to finally a carbon with no adjacent protonated carbon atoms.

Evidence for such has been given by Levy (47)

in the ^{13}C - T_1 data for mescaline- d_3 where the relaxation is dominated by the ^{13}C - ^1H dipole-dipole mechanism. The T_1 values and structure are shown in Figure 6. As expected C-1 has the shortest T_1 since it has two adjacent (ortho) protonated aromatic carbons and the adjacent CH_2 protons. The C-3,5 carbons have a much longer T_1 since they have only one adjacent protonated carbon. The longest T_1 will be for C-4 which has no adjacent protonated carbon atoms. Another example is the T_1 data for acenaphthene (48), Figure 6. The T_1 for C-8b is very long since the nearest proton is three bonds away. Both C-2a,8a and C-5a have adjacent proton-bearing carbons which markedly decrease their T_1 values by 35 to 41% of that of C-8b. It is also possible to distinguish between C-2a,8a and C-5a by noting that C-2a,8a has three geminal protons while C-5a has only two.

There is this same variety of types of quaternary carbons in phenanthrene (8) and its two deuterated analogues, 9 and 10. Thus, the T_1 values for C-4a,4b and C-8a,10a have been determined and are shown in Figure 7. The standard deviations for each value are given in parenthesis. The greater standard deviations for 9 and 10 are a consequence of the smaller amounts available of these compounds and the corresponding compromise on the total time and signal-to-noise ratio for their T_1 determination.

In phenanthrene (8) the resonance at $\delta = 132.03$ has a T_1 value which is significantly shorter than that of

Figure 6. The spin-lattice relaxation times (in seconds) for mescaline- d_3 (47) and acenaphthene (48).

Figure 7. The spin-lattice relaxation times (in seconds) for the quaternary carbons in phenanthrene and its deuterated analogues. The standard deviations for each value are indicated in parenthesis.

the $\delta = 130.30$ resonance --- greater than twice the standard deviation separates these T_1 values. This is consistent with what would be expected for $\delta = 132.03$ belonging to C-8a,10a and $\delta = 130.30$ to C-4a,4b, the assignments already determined. The C-8a,10a carbons have two adjacent protonated carbons each, while C-4a,4b have only one adjacent protonated carbon. Thus C-4a,4b will have a longer T_1 , as observed. In addition, due to this longer T_1 and the experimental conditions under which the spectra are obtained, it would be expected that the intensity of the C-4a,4b resonance would be less than that from C-8a,10a. This is observed in the ^{13}C NMR spectrum of phenanthrene (8).

The deuterated analogues of phenanthrene should also follow this reasoning. For 9,10-dideuteriophenanthrene (9) both the C-4a,4b and the C-8a,10a resonance should have similar T_1 's. This arises because C-8a,10a now has one of its adjacent hydrogen atoms replaced with a deuterium atom, and because the dipole-dipole coupling between ^{13}C and deuterium is only about 10% of that between ^{13}C and ^1H . Thus, both C-8a,10a and C-4a,4b only have one adjacent protonated carbon. The experimental T_1 values, as expected, are equal within the standard deviation (see Figure 7).

Similar arguments can be applied to 4,5-dideuteriophenanthrene (10). In this case, the T_1 value for C-4a,4b could not be accurately determined within a reasonable experimental time due to its great length. However, the data clearly indicated

that the C-8a,10a resonance relaxed substantially faster than that of the C-4a,4b one. The value for C-4a,4b shown in Figure 7 is estimated to be small, and is probably more like that of C-8b in acenaphthene. (shown in Figure 6) since in both cases the nearest proton is three bonds away. This value is still much longer than that for the peak assigned to C-8a,10a where the two adjacent protonated carbons should lead to more efficient relaxation and a shorter T_1 .

Thus, the ^{13}C T_1 data can be useful in making the assignments for quaternary carbons of polynuclear aromatic hydrocarbons. However, there are problems associated with this technique:

- (i) It cannot distinguish between quaternary carbons having the same number of adjacent proton-bearing carbons. It can only distinguish those types or groups of carbons which are in a different environment with regards to the number of hydrogen atoms two bonds away. For small molecules (such as those studied up to now) there are few quaternary centers and so this limitation is not so serious. The T_1 's could be used for unambiguous assignment just as in phenanthrene. However for larger polycyclic molecules, the number of quaternary carbons rises rapidly and the T_1 's will only separate out the various types of quaternary carbons but will not permit distinction and thus assignment within each type.
- (ii) One must assume that a unique correlation time, τ_C ,

applies to all carbons, i.e. the molecule rotates in an isotropic manner in solution. This is not a bad assumption for the molecules studied here. However, the larger polycyclic systems might be shaped such that their rotation in solution would be anisotropic. In this case, there will not be a unique τ_C and the method will have to be used with caution.

- (iii) The experimental time needed to determine these T_1 values is excessively long and discourages the routine use of this technique. Even using an efficient method like saturation recovery*, the experiment can take days to measure a T_1 in the order of 100 seconds with a modest signal-to noise ratio. To reduce this time a concentrated sample can be used, but this is generally not possible for the polynuclear aromatics where solubility is often a limiting factor.

*The saturation recovery method of T_1 measurement is more efficient time wise than the inversion recovery method when very long T_1 's are being measured (for the same signal-to-noise ratio in both). A 30% reduction in time can result since in saturation recovery there is no 4-5 $\times T_1$ pulse delay after each data acquisition (33).

These difficulties, however, must be viewed in the perspective that this technique is one of the few which can be applied to the non-protonated quaternary carbons. Selective proton decoupling and deuteration are not possible. In some cases secondary deuterium isotope shifts are the only clues when making the assignments, but these depend heavily on the possibility of deuterating neighboring carbon atoms. The T_1 method, on the other hand, requires no synthetic expertise.

The length of time for determining such T_1 values is a serious problem. However, it has been shown from the few compounds studied here that the signal intensity of a quaternary carbon bears a relationship to the T_1 value: for all resonances representing the same number of carbon atoms, and under the routine conditions used to gather the spectra, the more intense the peak, the shorter the T_1 (and vice versa). These estimates of T_1 are only relative and not absolute, and, due to the differences in intensity being small (e.g. see Figure 5), are dependent on a fairly high signal-to-noise ratio. While these cautions must be remembered, it remains possible to use this technique as a check or verification of quaternary carbon assignments which for the most part must come from model compounds. This method of estimating T_1 differences will be used to a very limited extent in

light of its approximate nature.

F. Assignment of the ^{13}C Chemical Shifts of Triphenylene (11) and the Dibenzanthracenes, 12-13. The Results for Table 6.

The chemical shifts for triphenylene (11) can be simply assigned and these current results in CDCl_3 agree well with those originally reported for the compound in CS_2 (43,49). There only is one quaternary carbon resonance, $\delta=129.72$, which is immediately assignable to C-4a,4b,8a,8b,12a,12b. The C-1,4,5,8,9,12 carbons are all in a position very much like the bay region of phenanthrene. It would be expected that the steric interaction of the pairs' C-4,5, C-8,9 and C-1,12 protons would cause an upfield resonance just as it did for C-4,5 in phenanthrene. The observed shift is $\delta=123.20$. The assignment of the remaining peak at $\delta=127.11$ to C-2,3,6,7, 10,11 is reasonable using C-2,3,6,7 in phenanthrene as a model (within 0.6 ppm of each other).

The triphenylene molecule serves as a useful model for part of dibenz[a,c]anthracene (12). The C-1 to C-8 region of this compound is very similar to triphenylene (11); this observation is reinforced by the similarity of the actual chemical shift values (all are within ± 0.46 ppm). Thus, C-1,8 and C-4,5 are assigned

to the $\delta=123.41$ and $\delta=123.66$ pair just as C-2,7 and C-3,6 are assigned to $\delta=127.57$ and $\delta=127.44$. Due to the small chemical shift differences within each pair it is not possible to make the absolute assignment. The proton spectrum of this compound remains unanalysed but the expected singlet for the protons on C-9,14 can be easily observed at $\delta=9.04$. This ^1H NMR resonance is the furthest downfield signal and so single frequency selective proton decoupling was carried out. This unambiguously shows $\delta=122.03$ to be the C-9,14 resonance in the ^{13}C NMR spectrum. It is interesting to note that these carbons resonate ca. 4 ppm to higher field than C-9,10 of anthracene (5). This is presumably due to the same steric interaction which was noted for C-4,5 in phenanthrene (8) and for C-4,5, C-8,9 and C-11,12 in triphenylene (11). The assignment of C-10,13 and C-11,12 in dibenz[a,c]anthracene is made by analogy to the C-1,4,5,8 and C-2,3,6,7 signals in anthracene (5). Both of the observed values are close to those predicted from this model.

The assignment of the quaternary carbons presents more of a problem. The C-4a,4b and C-8a,14b resonances should be much like the one quaternary resonance of triphenylene. The assignments, $\delta=130.10$ and $\delta=130.19$, are slightly downfield (≈ 0.4 ppm) from that in triphenylene but this is the same amount that the C-1,4,5,8 and C-2,3,6,7 are downfield from the corresponding triphenylene resonances.

Again, absolute assignment of C-4a,4b and C-8a,14b is not possible due to the small separation of the resonances. The C-8b,14a resonance should be shifted upfield from that of 11 due to the ring addition. Using the quaternary carbon of anthracene (5) and naphthalene (1), one calculates that the ring addition should cause a 1.91 ppm upfield shift. The prediction agrees well with the observed value of $\delta=128.49$. The C-9a,13a chemical shift, $\delta=132.28$, is assigned on the basis of anthracene as a model.

The intensities of the four quaternary carbon signals in dibenz[a,c]anthracene (12) should help identify that belonging to C-9a,13a. These carbons, due to an anticipated smaller T_1 because of their two adjacent protonated carbons, should have a greater intensity than the other three signals. These three should have less but equally intense signals. Figure 8 shows the spectrum of 12 and, as expected, three resonances are of equal intensity and one is 30% taller. This observation agrees with the prior assignment by use of model compounds of this peak to C-9a,13a.

Due to favorable symmetry and solubility in CDCl_3 , the actual T_1 values of these four carbons were determined. In this way it was hoped to verify these results and build confidence in using quaternary carbon

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Figure 8. ^{13}C NMR spectrum of dibenz[a,c]anthracene and the T_1 values (in seconds) for the quaternary carbon resonances. The standard deviations for each value are indicated in parenthesis.

intensities as a means of assignment. The T_1 values of the four resonances are shown in Figure 8. They confirm the assumption made about the T_1 values based on intensities, and clearly permit the C-9a,13a assignment to be made with confidence.

The assignment of dibenz[a,h]anthracene (13) is more difficult since neither of the other two previous compounds in this table can serve as models. Fortunately, the ^1H NMR spectrum has been completely assigned (13,15), and in it the protons of C-7,14 ($\delta=9.13$, a sharp singlet) and of C-1,8 ($\delta=8.85$, a doublet) appear downfield from the other resonances. This permits selective ^1H decoupling, and C-7,14 and C-1,8 are unambiguously assigned to $\delta=122.11$ and $\delta=122.83$, respectively. The remainder of the assignments rely on phenanthrene (8) as a model compound. This would seem reasonable since dibenz[a,h]anthracene looks like two molecules of phenanthrene condensed end to end, and the effect of ring addition decreases quickly over a few rings. The assignments of C-1,8 and C-7,14 verify this since they are very much like C-4,5 of 8. Thus, it is expected that C-4,11, C-2,3,9,10 and C-5,6,12,13 will have chemical shifts like C-1,8, C-2,3,6,7 and C-9,10, respectively, of phenanthrene. The observed value for C-4,11 of 13, $\delta=128.57$, is nearly identical to the model shifts (which is

reasonable since it is so far removed from the differences between 13 and 8). In each of the two remaining pairs of resonances the absolute assignment cannot be made due to the small separation between the two peaks.

The same model compound, phenanthrene, can be invoked to assign the quaternary carbons of dibenz[a,h]-anthracene (13). It is expected that C-4a,11a will have a chemical shift much like C-8a,10a in 8 and thus they are assigned to $\delta=132.03$. Analogously, C-7b,14b of 13 are given a value of $\delta=130.21$ since they should be most like C-4a,4b of 8. This is also very close to the value expected using C-8a,14b of 12 as a model. Using C-8b,14a of this same compound, 12, the C-7a,14a carbons of 13 were assigned to $\delta=129.09$. Again, an upfield shift is noted for this quaternary carbon in the bay region. The C-6a,13a carbons of 13 were assigned to the remaining resonance at $\delta=130.77$. If C-9a,13a of 12 is used as a basis ($\delta=132.28$), and if the difference between a quaternary carbon in naphthalene and the C-8a,10a quaternary carbon of phenanthrene is a 1.5 ppm upfield shift, the predicted chemical shift of C-6a,13a of 13 is $\delta=130.78$. This is seen to be in good agreement with the observed value. It is noteworthy that the two lowest field quaternary carbon resonances in the spectrum of 13 are 30% more intense than the two higher field ones.

This is expected since the lower field resonances have been assigned to C-4a,11a and C-6a,13a. Using number of neighboring protonated carbons and T₁ arguments, the resonances of these carbons should be more intense than those of C-7a,14a and C-7b,14b.

G. Assignment of the ^{13}C Chemical Shifts of Benz[a]anthracene and its Methylated Derivatives, 14 - 17.

The Results for Table 7.

As previously detailed, the compounds in this table, benz[a]anthracene (14), 7-methylbenz[a]anthracene (15) and 7,12-dimethylbenz[a]anthracene (17), are an important group of carcinogenic compounds.

In making the ^{13}C chemical shift assignments it will be important to assign correctly benz[a]anthracene (14) as it will be extensively used as a model compound in making the assignments of its methylated derivatives, 15 and 17.

The single frequency selective proton decoupling can be used to assign unambiguously three of the ^{13}C resonances of benz[a]anthracene (14). Analysis of the proton NMR spectrum of 14 in CS_2 has been published (13,15), and clearly allows assignment of $\delta=9.08$, $\delta=8.75$ and $\delta=8.27$ to H-12, H-1 and H-7, respectively, for 14 in CDCl_3 . The good separation between these and other resonances permits the selective decoupling technique to assign $\delta=121.42$, $\delta=122.83$ and $\delta=127.21$ to C-12, C-1 and C-7, respectively. This assignment of C-1 is in excellent agreement with C-1 of 13 which should be a good model for it. Again the γ interaction between C-1 and C-12 in 14 causes their upfield shift..

The C-2 and C-3 carbons of 14 should be identical (or nearly identical) just as C-2,3,6,7 in phenanthrene (8) or C-2 and C-3 in dibenz[*a,h*]anthracene (13). They are assigned to $\delta=126.71$ and $\delta=126.65$, but a distinction between them cannot be made unambiguously due to the closeness of their chemical shifts. The resonance of double intensity at $\delta=126.95$ is assigned to the K-region carbons C-5 and C-6. The C-9,10 resonance of phenanthrene (8) was used as a model compound for this. The C-9 and C-10 carbons of 14 are well removed from the fourth aromatic ring angularly attached to the three-ring anthracene portion. It is reasonable to expect C-2,3,6,7 of 5 to be a good model for these carbons. There are only two unassigned peaks remaining in this region, $\delta=125.56$ and $\delta=125.67$, which, therefore, can be readily assigned to C-9 and C-10. Further, the H-9 and H-10 protons appear at the highest field position (13,15) in the ^1H NMR. Single frequency selective proton decoupling is not possible due to the proximity of a number of multiplets near the H-9 and H-10 multiplet. However, the two carbon resonances assigned to C-9 and C-10 sharpen to near singlets as the ^1H decoupling frequency is passed from high field through

the H-9 and H-10 multiplet region in the proton spectrum.

The remaining three protonated carbon resonances must be assigned on the basis of model compounds alone. Just as C-1, C-2, C-3, C-5 and C-6 of 14 have been assigned on the basis of the results for phenanthrene (8), C-4 should also be possible. Accordingly, $\delta=128.49$ is assigned to C-4, a value near that of C-1, 8 in 8. The C-1,4,5,8 resonance of anthracene (5) should be a suitable model for C-8 and C-11 of 14. Although it is not possible to make a definite assignment of these resonances to each of the two carbons, the value most near that of anthracene's, $\delta=128.32$, presumably belongs to C-8 since it is furthest removed from the fourth ring. There may be some shifting of the C-11 resonance due to a secondary steric effect resulting from the strong γ interaction of the protons on C-1 and C-12.

The quaternary carbons of benz[a]anthracene (14) are assigned in the following manner. The one double intensity quaternary carbon resonance, $\delta=131.89$, is assigned to C-4a,6a using the C-8a,10a of phenanthrene (8) as a model. Just as C-5 and C-6 had an identical spectral position, so do their neighboring carbons, C-4a and C-6a. For C-12a and C-12b, the C-14a and C-14b of dibenz[a,h]anthracene (13) should be good models

(just as C-4a of 13 correctly predicts the value for C-4a and C-6a of 14). Using this model compound, C-12a and C-12b are assigned to $\delta=128.79$ and $\delta=130.47$, respectively. The value of C-12b is close to C-4a,4b of phenanthrene (8) and that of C-12a shows the 1.5 ppm upfield shift (from that in phenanthrene)^c which results from linear ring addition*. These assignments are confirmed by the observed intensities of all these quaternaries. Only the two upfield ones at $\delta=130.47$ and $\delta=128.79$ have reduced intensities compared to the others. Since C-12a and C-12b have only one adjacent protonated carbon each, it is reasonable that these are assigned to the two resonances with reduced intensities. The other four must belong to the remaining quaternaries having two adjacent protonated carbons and thus shorter T_1 values and greater resonance intensities under the spectral conditions used.

* See the explanation given in the assignment of the quaternary carbons of 12 and 13. It is to be noted that the results for C-11a and C-12a of 14 are reversed in reference (40).

The C-7a resonance should be very much like the quaternary carbon in anthracene (5) and for this reason is assigned to $\delta=131.85$. The remaining resonance at $\delta=130.56$ is that of C-11a. Although this is at a different position than the corresponding quaternary carbon of anthracene, it shows the same upfield shift as C-11 showed.. This is most likely due to the steric interaction between C-1 and C-12 which seems to cause C-11 and C-11a (and C-10 slightly) to lose their anthracene-like character.

In 7-methylbenz[a]anthracene (15) selective proton decoupling and use of benz[a]anthracene (14) as a model permits most of the shifts to be readily assigned. The analysis of the ^1H NMR spectrum has been published (15), and this readily shows that H-1 and H-12 ($\delta=8.80$ and $\delta=9.06$, respectively) are suitably simple and separate from other resonances to permit selective single frequency proton decoupling. This established the resonances $\delta=123.64$ and $\delta=120.30$ as belonging to C-1 and C-12, respectively. The 1.12 ppm upfield shift of

C-12 is comparable to the 0.82 ppm upfield shift for C-10 of anthracene (5) after methylation at C-9 to form 9-methylanthracene (7).

The position of C-2, C-3 and C-4 in 15, all in the ring furthest from the methylation, should not be greatly changed from that in 13 or 14. Accordingly, the double intensity peak at $\delta=126.82$ is assigned to C-2,3, and that at $\delta=128.36$ to C-4. The remaining protonated carbon resonances are assigned using the chemical shifts of benz[a]anthracene (14) as a basis. The effect of methylation, as predicted by the corresponding positions in anthracene (5) and 9-methylation (7), is added to the base shift in 14 to give the final predicted value. Considering C-1,8 between 5 and 7, one expects a 3.6 ppm upfield shift, and thus C-6 and C-8 of 15 are assigned to $\delta=123.09$ and $\delta=124.46$, respectively (both are within 0.3 ppm of the predicted value). For C-9 and C-10 in 15, the model system predicts one resonance should remain the same and one should decrease by 0.45 ppm from the benz[a]anthracene value. The two resonances $\delta=125.67$ and $\delta=125.24$ fulfill these conditions and are assigned to C-9 and C-10 respectively. Just as C-9 should remain unshifted in 15, so should C-5 which occupies the

symmetrical position to C-9 on the other side of the methyl addition. Thus, C-5 is assigned to $\delta=126.94$, the same chemical shift as in the benz[a]anthracene model. The one remaining resonance at $\delta=129.76$ is assigned to C-11. The corresponding carbon in 7 is at $\delta=129.05$, adding validity to the assignment.

The quaternary carbons of 7-methylbenz[a]-anthracene are assigned on much the same basis as the preceding protonated ones. Using the arguments of corresponding carbons in 5 and 7, and the observed effect of the methyl in 15 on C-5, C-9 and C-10, C-4a is assigned to $\delta=131.68$, a value very much like C-8a, 10a in phenanthrene. The resonances for C-7, C-6a, C-12a and C-12b in 15 are predicted utilizing C-9, C-8a, 9a, C-4a, 10a and C-4,5, respectively, in 5 and 7. Thus, the chemical shifts in 14 should undergo: for C-7, a 3.91 ppm upfield shift; for C-6a, a 1.44 ppm upfield shift; for C-12a, a 0.10 ppm upfield shift; and for C-12b, a 0.71 ppm downfield shift. All the observed shifts are within 0.2 ppm of those predicted; $\delta=130.87$, $\delta=130.46$, $\delta=128.64$ and $\delta=131.01$ for C-7, C-6a, C-12a and C-12b, respectively. For C-7a and C-11a, just

as for C-7 and C-11, use of the benz[a]anthracene chemical shifts as a starting point is not practical since this end of 15 is well removed from the angularly attached ring. Instead these chemical shifts are made directly on the basis of 9-methylanthracene (7) itself. Accordingly, C-7 is assigned to $\delta=129.76$ and C-11a to $\delta=131.53^*$.

It is interesting to note in 7-methylbenz[a]-anthracene that the T_1 argument used for verifying quaternary carbon assignments is again valid. Only two carbons in 15, C-4a and C-11a, have two adjacent protonated carbons. One predicts and observes only two quaternary carbon resonances which are more intense than the others. These two lowest field peaks are those which have already been assigned to C-4a and C-11a. This adds validity to the assignments made.

The chemical shifts for 1,8-dimethylnaphthalene (16) have been previously reported by Jones et al. (50)

* These results for the quaternary carbons of 15 differ somewhat from those in reference (40).

and Wilson and Stothers (35). The results of the latter are included in Table 7 since 16 is a very important model for the δ steric interactions in 7,12-dimethylbenz[a]anthracene (17). These chemical shifts have been obtained from a dilute CDCl_3 solution and the assignments made by the single frequency selective proton decoupling technique.

The assignment reasoning for 7,12-dimethylbenz[a]anthracene (17), is now presented. For the proton on C-1 the ^1H NMR spectrum shows a distinct low field multiplet at $\delta=8.23$. This permits use of selective proton decoupling to determine that $\delta=130.25$ is the C-1 resonance. This represents a 6.61 ppm downfield δ shift due to the strong steric interaction between the protons of C-1 and those of the methyl group on C-12. The deshielding δ effect noted for 17 is another example of the large downfield shift which have been found for compounds containing spatially interacting δ functions (51,52). A good example of the same effect is found in the chemical shifts of the methyl groups in 1-methylnaphthalene (3) and 1,8-dimethylnaphthalene (16). In 16 the two methyl

groups have a δ relationship and compared to 1 their chemical shift is 6.7 ppm downfield (vide infra.)

The assignment of C-2 and C-3 in 17 is made to $\delta=126.34$ and $\delta=126.41$, although not unambiguously, on the basis of the same carbons in 14 and 15. One would have expected C-4 of 17 to resonate at the same position, as that of C-4 in 15 and to be relatively unaffected by the addition of another methyl group. However, it must be assigned to $\delta=127.43$, the only remaining resonance in the expected region. This represents a 0.93 ppm upfield shift. Using C-1,4,5,8 and C-2,3,6,7 of 9,10-dimethylanthracene (6), it is possible to assign C-8 and C-11, and C-9 and C-10, respectively, of 17. While total unambiguous assignment is not possible within each group, the chemical shifts are very near the corresponding values in 6 due to their isolation from the additional, angularly-attached aromatic ring in 17. On the other hand, assignment of C-5 and C-6 in 17 is best made by using benz[a]anthracene as a base and adding on the effect of the methyl groups. Using C-1,4,5,8 of anthracene (5) and of 9,10-dimethylanthracene (6), the effect of the dimethyl substitution

on C-6 should be a 3.05 ppm upfield shift. Starting with C-6 of 14, one predicts C-6 in 17 to have a chemical shift of $\delta=123.90$ and observes one at $\delta=123.77$. Alternatively, one could have predicted a value of $\delta=123.80$ by using the value of C-6 of 7-methylbenz[a]-anthracene as a basis and by estimating the effect of methyl addition from anthracene (5) and 9-methyl-anthracene (7). The same two model systems predict an upfield shift for C-5 in 17 and it is assigned to the remaining resonance at $\delta=125.50$.

The quaternary carbons of 7,12-dimethylbenz[a]-anthracene are difficult to assign. The best approach for a number of the quaternary carbons is to use the 7-methylbenz[a]anthracene (15) as a base and to calculate the effect of the methyl addition from 1-methylnaphthalene (3) and 1,8-dimethylnaphthalene (16). These two compounds are used since they contain the strong δ steric interaction which is very prevalent in 17, as well as accounting for the methyl addition. It is recognized that in 17 the molecular arrangement is more rigid and that the strong steric interaction is not as relieved by bond angle distortion as it is in 16. Nevertheless, by observing the resonance positions of C-8a, C-1, C-2 and C-8 in 3 and 16, the chemical

shift of C-12a, C-12b, C-4a, and C-12, respectively, of 17 can be predicted by adding the observed effect to the chemical shift of the same carbon in 15. There is one double intensity quaternary carbon resonance in 17, $\delta=129.09$, which is assigned to C-7a, 11a. It is expected that this region of the molecule is sufficiently removed from the angularly attached ring so that the two carbons will have a similar chemical shift and one like that of C-4a, 8a, 9a, 10a in 9,10-dimethylantracene (6). The observed double intensity resonance is in fact within 0.84 ppm of this model chemical shift. The validity of 6 as a good model for this end of 17 is reinforced by the C-8, C-9, C-10 and C-11 resonances of 17 being very similar to those of C-1, C-2, C-3 and C-4 of 6. By noting that the position of C-8a, 9a remains relatively unchanged in 6 and 7, the assignment of C-6a in 17 is made to $\delta=130.49$, similar to that of C-6a in 15. The remaining quaternary carbon, C-7, is assigned to $\delta=127.74$ on the basis of C-7 in 15 and the large upfield shift noted at C-9 in 6 and 7.

Only two quaternary carbons in 17 are expected to show an intensity different from the others. For C-4a there are two adjacent protonated carbons, while

for C-12a there are none (the remaining carbons all have one). Thus, these two carbons should have the smallest and largest T_1 's of the quaternary carbons, and therefore the largest and smallest resonance intensity, respectively. It is a verification of the model compounds that this is observed in the spectrum of 17. The C-12a resonance shows this dramatically by being 50% the intensity of the other quaternary carbon resonances. The remaining quaternary carbons with one adjacent protonated carbon all have the same intensity to within 5-10% of each other.

H. Assignment of the ^{13}C Chemical Shifts of 3-Methylcholanthrene, 20. The Results for Table 8.

The first two compounds in Table 8 are acenaphthene (18) and 2-methylnaphthalene (19). The ^{13}C chemical shifts for these compounds have been previously reported and discussed; (50,53,54) for 18 and (35,36) for 19. The results reproduced in this table are for 18 and 19 as a dilute solution in CDCl_3 and are taken from (55) and (35), respectively. These two compounds are very important models which will be used in making the assignments of 3-methylcholanthrene (20).

The chemical shift assignments in 3-methyl-cholanthrene (20) are easier to make, by ~~any~~ technique, because of its larger chemical shift range, as illustrated in Figure 9. The ^1H NMR spectrum of 20 in CS_2 has been analysed (15). This spectrum shows three protons which are sufficiently isolated to allow selective proton decoupling of the corresponding carbon resonances. The hydrogen on C-6 appears as a singlet at $\delta=8.75$, which upon decoupling indicates that $\delta=116.32$ is that belonging to C-6. The proton spectrum also shows an isolated doublet for H-4 and H-7 at $\delta=7.65$ and $\delta=8.63$, respectively. The use of these in the selective decoupling technique allows the resonances at $\delta=130.74$ and $\delta=123.29$ to be assigned to C-4 and C-7, respectively.

Just as they are used to assign the remaining carbons, model compounds could have been used to predict successfully the chemical shifts of C-6 and C-4. For these two carbons, the C-12 and C-10 resonances, respectively, of benz[a]anthracene (14) are used as a basis. To these must be added the effect of the methyl addition at C-3 and the formation of the five-membered ring in 20. The effect of the methyl group at any position is assessed by subtracting the chemical shift of the appropriate carbon of naphthalene 1 from that of the same carbon in

Figure 9. ^{13}C NMR spectrum of 3-methylcholanthrene (20)

2-methylnaphthalene (19). In determining the effect of the formation of the five membered ring the chemical shift of the appropriate carbon in naphthalene 1 is subtracted from that of the same carbon in acenaphthene 18. The orientations of the molecules 1, 14, 18 and 19 in relationship to that of 20 in Figure 3 demonstrate the model system. The predicted values for C-6 and C-4 of 20 are $\delta=115.1$ and $\delta=129.3$. Another model system for C-4 in 20 involves using C-4 of 18 as a basis and calculating the effect of methyl substitution from 1 and 19.

By invoking either of the model systems used to estimate C-4, it is possible to assign C-5 in 3-methylcholanthrene (20). The predicted values of $\delta=121.5$ and $\delta=121.3$ agree with the assigned value of $\delta=122.82$. For C-8 and C-9 of 20 two closely spaced resonances, $\delta=126.56$ and $\delta=126.44$, are collectively assigned on the basis of C-2 and C-3 of 14 or 15. The C-4 resonance of 14 or 15 serves as the model for C-10 in 20, and $\delta=128.65$ is assigned to this carbon. These chemical shifts of 20 are closely approximated by the model compounds since their carbons are far removed from any of the substituents on the benz[α]anthracene core of 20. For C-11 of 20 these considerations are no longer strictly true, as indicated by the assigned resonance $\delta=125.86$, a 1 ppm shift to higher field than the C-5 model position in 14 or 15.

The one remaining protonated aromatic carbon, C-12, is assigned to $\delta=123.76$ on the basis of C-6 of 7-methylbenz[a]anthracene (15). For both of these there is a saturated carbon at the position and hence the observed upfield shift relative to the same position in benz[a]anthracene. The two saturated carbons of the five-membered ring in 20, C-1 and C-2 are assigned to $\delta=29.73$ and $\delta=29.69$. These values are only 0.5 ppm from the C-1 and C-2 of 13, and show very little effect of any steric interaction with the methyl group at C-3 or the C-12 position of 20.

The quaternary carbons of 3-methylcholanthrene (20) are also assigned with these same model compounds. The C-2a, C-12b and C-12c carbons are all assigned on the basis of the chemical shifts of C-2a, C-8a and C-8b of acenaphthene (18). To these are added the effect of addition of the methyl at C-3, as calculated from a comparison of naphthalene (1) and 2-methylnaphthalene (19). The predicted values, $\delta=144.4$, $\delta=145.1$ and $\delta=139.0$ for C-2a, C-12b and C-12c, respectively, are all just slightly downfield from the observed values of $\delta=142.17$, $\delta=143.22$ and $\delta=137.75$. Using the same arguments and the C-3 in 18 as a reference point, the predicted value for C-3 in 20 is $\delta=128.3$, while the observed value is $\delta=128.34$. This assignment of C-3 is also predicted ($\delta=128.1$).

by using C-9 of 14 as a reference and adding the effects of methyl substitution (using C-2 of 1 and 19) and ring formation (using 1 and 18).

This same approach, using the chemical shifts of benz[a]anthracene as a basis and adding the effects of the methyl and ring formation where necessary, allow the remaining carbons of 20 to be assigned. The C-10a is assigned to $\delta=131.93$ on the sole basis of C-4a in 14 since it is so far removed from other possible effects. For C-5a, C-6a and C-12a of 20 the two aforementioned effects will alter the values in 14. Using carbons in 1 and 19, and in 1 and 18 to evaluate the effects, the predicted values for the three carbons are $\delta=127.3$, $\delta=129.5$ and $\delta=124.9$. The observed values for C-5a, C-6a and C-12a are $\delta=127.43$, $\delta=129.92$ and $\delta=125.36$; respectively. The remaining resonance, $\delta=131.70$, is assigned to C-6b. No suitable model exists for this region, but this value is near that of C-12b in 7-methylbenz[a]anthracene (15), which most closely approximates the existing situation.

The T_1 intensity argument supports these assignments of the quaternary carbons. The lowest intensity should be for C-12c which is removed from any proton-bearing carbons (by two bonds) and thus will

have a very long T_1 value. The C-5a and C-10a, and C-2a and C-12b resonances should have shorter T_1 values and greater intensities due to the presence of two adjacent proton-bearing carbons or two protons on one adjacent carbon, respectively. These predictions are observed in the spectrum, Figure 9. This T_1 argument is valuable especially for C-10a, $\delta=131.93$, which could have also been assigned to $\delta=131.70$ on the basis of model compounds. However, this resonance is greatly reduced in intensity in relation to the former. The remaining resonances are much the same in intensity and cannot be used to assign any of the quaternary carbons.

I. Assignment of the ^{13}C Chemical Shifts of Benzo[a]pyrene, 22-23. The Results for Table 9.

The ^{13}C chemical shifts of pyrene (21) determined in the current study are shown in Table 9. These assignments were based on those of previous reports (30,53, 56,57) which were made with techniques such as ^{13}C labelling and analysis of ^{13}C - ^{13}C coupling constants. The same assignments for 21 were independently determined by invoking model compound chemical shifts and resonance intensity

arguments similar to those used for other compounds in the current work.

The assignment of the ^{13}C NMR spectrum of benzo[a]pyrene (22) is made using a variety of techniques. Many of the chemical shifts can be predicted using pyrene (21) as a reference and adding to it the effects calculated from simpler compounds (i.e., 1, 5, 8, 14). The ^1H NMR spectrum has been completely analysed (13,15,16,23,58) and, where a resonance is well separated from the rest, single frequency selective proton decoupling leads to unambiguous assignments in the ^{13}C spectrum. Just as phenanthrene (8) was assigned by using deuterated analogs (9 and 10), benzo[a]pyrene (22) and 1,3,6-trideuteriobenzo[a]pyrene (23) can be used in a similar manner. The upfield isotope shifts, couplings and intensity changes which will be noted between 22 and 23 have previously been discussed in detail (see section E for the assignment of the phenanthrenes).

When the ^{13}C spectrum of benzo[a]pyrene (22) was first obtained, it was difficult to know if the two low intensity absorptions at $\delta=125.27$ and $\delta=123.58$ were real (see Figure 5). To verify that these were in fact quaternary carbon resonances of 22, a spectrum of 22 in the presence of $5 \times 10^{-2} \text{ M Cr(acac)}_3$ was

obtained. This paramagnetic relaxation reagent is known (24-26) to reduce greatly spin-lattice relaxation times and thus cause enhancement of signals. The two suspect absorptions were now found to have an intensity equal to that of the protonated carbons. This verifies that the two low intensity resonances are those of quaternary carbons.

Using the assignments of (16) for the ^1H NMR spectrum of benzo[a]pyrene, the hydrogens of C-6, C-10 and C-11 of 22 were sufficiently isolated to allow selective proton decoupling. The C-6 proton gives a sharp singlet at $\delta=8.43$ and irradiation of this resonance indicated the ^{13}C resonance at $\delta=125.38$ to be that of C-6. The C-10 and C-11 hydrogens give one lowfield multiplet centered at $\delta=8.96$, and when the proton decoupler is placed at this position, C-10 and C-11 can be assigned to $\delta=122.87$ and $\delta=122.00$, respectively. As with the carbons in the bay region of 8, 11, 12, 13, 14 and 15, the C-10 and C-11 carbons of 22 are shifted upfield due to the same γ steric interaction. The protons of the carbons in such a rigid geometrical relationship interact very strongly and lead to these steric shifts. The selective decoupling technique does not give an unambiguous assignment of these two peaks but the one at $\delta=122.87$ is assigned to C-10 since it should be most like C-4,5 of phenanthrene (8).

The remaining chemical shift assignments have

been made using model compounds and additivity relationships. To verify these, 1,3,6-trideuteriobenzo[a]pyrene (23) was synthesized. Using the known isotope shifts (40,44) the consistency of the assignments of 22 was checked. The most visually obvious effect of deuteration occurs for the resonance of the carbon on which substitution occurs and thus, the assignments of C-1, C-3 and C-6 are readily made. The resonance at $\delta=125.38$ in 22, previously assigned by selective decoupling to C-6, behaves as expected in 23 by becoming a very low intensity triplet shifted 0.32 ppm upfield. In addition, comparison of the spectrum of 22 and 23 shows that both $\delta=124.61$ and $\delta=124.68$ are absent in 23 and in their place are low intensity triplets which are both shifted 0.31 ppm upfield. These two peaks are assigned to C-1 and C-3 (there is no way to distinguish one from the other and so the assignments for C-1 and C-3 could be inter-changed in Table 9). These values are close to what one would expect using the C-1,3,6,8 resonance of pyrene (22), $\delta=125.06$, as a model.

The C-2,7 resonance of 21 serves as an excellent model for C-2 in 22, a carbon far removed from the added ring. The absorption at $\delta=125.78$ is assigned to C-2 of benzo[a]pyrene (22) and is verified by its behavior in the deuterated derivative 23.

There, it has broadened and shifted upfield by 0.11 ppm, indicative of β deuterium substitution. Martin *et al.* (44) have found a β deuterium isotope shift of 0.12 ppm for protonated carbons, and it is interesting to note here that the observed effect on C-2 in 23 of the two β deuterium atoms indicates that these isotope shifts are not cumulative.

The C-4 and C-5 of 22 should be similar to the C-4,5,9,10 resonance of 21 ($\delta=127.50$). There are two peaks in the spectrum of 22, $\delta=127.33$ and $\delta=127.66$, which are equally and oppositely displaced from this chemical shift. It is possible to predict the values for C-4 and C-5 by using a reference value from 21 and considering the resonance positions of C-2,3,6,7 and of C-1,4,5,8, respectively, in 1 and 5. These two compounds predict for C-4 and C-5 of 22 the effect of adding another benzenoid function to the pyrene nucleus, 21. For C-4, the C-2,3,6,7 in 1 and 5 predict a small upfield shift, and thus it is assigned to $\delta=127.33$. In a similar manner, C-1,4,5,8 of 1 and 5 predicts a small downfield shift, and C-5 in 22 is assigned to $\delta=127.66$. To verify these, it is noted in 23 that C-4 and C-5 are in a position to receive a peri γ deuterium isotope shift, which Martin *et al.* (44) show to be ca. 0.04 ppm.

upfield. In 23 the two resonance assigned to C-4 and C-5 are noted to have undergone a 0.05 ppm upfield shift.

The peak at $\delta=128.77$ is assigned to C-7 of benzo[a]pyrene(22) on the basis of C-8 in benz[a]anthracene (14) ($\delta=128.32$). Using C-1 of naphthalene (1) and phenanthrene (8) to estimate the effect of adding a ring to the benz[a]anthracene nucleus, a further 0.59 ppm downfield shift is added to predict C-7 of 22 at $\delta=128.91$. As with C-4 and C-5 of 22, C-7 is in a peri γ position to the deuterium at C-6 in 23. Accordingly, it is noted to have undergone a 0.06 ppm upfield shift in 23.

For C-12 of 22, the resonance at $\delta=128.00$ is assigned using that of C-4,5,9,10, of 21 as a starting point. The effect of condensing another ring onto the pyrene nucleus is most easily visualized and estimated by considering C-3 in 1 and 8. The observed 0.66 ppm downfield shift leads to a predicted value of $\delta=128.16$ for C-12 of 22. As expected, the peri γ deuterium isotope shift causes a 0.05 ppm upfield shift of this resonance in 23.

The two remaining protonated carbon resonances of 22, $\delta=125.88$ and $\delta=125.91$, are assigned to C-8 and C-9 on the basis of C-9 and C-10 of 14. Since these carbons are far removed from any deuterium atoms in 23, they remain

as predicted, unshifted in 23.

The quaternary carbon resonances of 22 can be assigned by using the same approach. The resonances for C-12b and C-12c in benzo[a]pyrene are expected to be like that of C-10b,10c in 21 ($\delta=124.96$). The effect of the ring addition on C-12b and C-12c of 22 is estimated by comparing C-4b of 8 with C-12b of 14, and C-4a of 8 with C-12a of 14, respectively. For the C-12b position of 22, these models predict a 0.17 ppm downfield shift of the pyrene resonance to $\delta=125.13$, and for the C-12c position a 1.51 ppm upfield shift to $\delta=123.45$. These agree well with the observed values of $\delta=125.27$ for C-12b and $\delta=123.58$ for C-12c. Neither of these resonances are observed in 23 because of broadening due to vicinal coupling to deuterium at C-1 and C-3 (for C-12b), and a broadening (due to vicinal coupling) and a coincident position with the C-6 triplet (for C-12c). A verification of these assignments can be found in their intensities, as shown in Figure 5. They were so low that it took addition of $\text{Cr}(\text{acac})_3$ to verify them. This would be expected for the resonances of C-12b and C-12c since they are so far removed from protonated carbons that their T_1 values will be excessively long and thus their intensities will be very low.

The quaternary carbons at C-3a and C-12a of 22 are assigned to $\delta=131.21$ and $\delta=131.27$ (no distinction can be made between them) using the C-3a,5a,8a,10a resonance ($\delta=131.21$) of 21 as a model. This same resonance provides the basic chemical shift for C-5a in 22. To this must be added the effect of the benzenoid ring addition; this is estimated using C-4a in naphthalene (1) and anthracene (5) (just as they were used for C-4 and C-5 in 22). The predicted, $\delta=129.39$, and the observed, $\delta=129.71$, values are reasonable. For C-6a in benzo[a]pyrene, the only possible model is C-7a in 14, and thus it is assigned to $\delta=131.46$ on this basis.

Two facts can be used to verify the assignment of these last four resonances to C-3a, C-5a, C-6a and C-12a in benzo[a]pyrene (22). Firstly, the intensities of these four lowest-field absorptions are near equal and are the largest* of the quaternary carbon resonances, as shown in Figure 5.

* The other two resonances at $\delta=128.10$ and $\delta=127.22$ have near the same peak height as these four resonances but this is due to their position on the wings of protonated carbon resonances. If an imaginary baseline is put in for these two resonances, their area is considerably less than that of these other four absorptions.

All of these assigned carbons, C-3a, C-5a, C-6a and C-12a have two adjacent protonated carbons, and thus the same predicted T_1 value. This should lead to four quaternary resonances of equal and greater intensity than the others, as observed. Secondly, all the carbons in question are β to a deuterium atom in 23. From the work of Martin *et al.* (44) this should lead to upfield isotope shifts of 0.07 ppm. Comparing the four resonances between the spectra of 22 and 23, all undergo a 0.07 to 0.09 ppm upfield shift in the deuterated compound. While these two methods do not verify individual assignments, they are valuable in checking groups of them.

The remaining quaternary carbons of 22, C-10a and C-10b are assigned to $\delta=127.22$ and $\delta=128.10$, respectively. For C-10a, the C-11a of 14 ($\delta=130.56$) is used as basis and a 3.23 ppm upfield shift is calculated from C-4a of 1 and 8, the same two model compounds used to predict the effect of this same ring addition on C-7, C-8 and C-9 in 22. The C-10b carbon of 22 is predicted to undergo the same 3.23 ppm upfield shift. However, the starting-point chemical shift is C-8a of pyrene (21), for which $\delta=131.30$. The predicted values for C-10a and C-10b of 22, $\delta=127.33$ and $\delta=128.07$, agree closely with the observed values. As previously mentioned, the intensities of these two resonances are less than those of C-3a, C-5a, C-6a or

C-12a since C-10a and C-10b have only one adjacent protonated carbon, and thus a larger T_1 and a decreased intensity, as shown in Figure 5. The resonances assigned to C-10a and C-10b show no isotope shift in 23, in agreement with their predicted behavior.

J. Assignment of the ^{13}C Chemical Shifts of Large Polynuclear Aromatic Hydrocarbons, 24-27. The Results for Table 10.

This table contains the ^{13}C chemical shifts for the aromatic carbons of the remaining compounds studied. The first two, perylene (24) and 1,12-benzoperylene (25), are structurally related and the complete results are presented for their carbons. The last two compounds, anthanthrene (26) and dibenzo[a,e]pyrene (27) are barely soluble in CDCl_3 . Even with the addition of $\text{Cr}(\text{acac})_3$ the quaternary carbons are not visible, and so for 26 and 27 only the chemical shifts of the protonated carbons are presented. Whereas various techniques are used in assigning 24, 25 and 26, only model compounds can be used to make the assignments in 27. Chronologically, the chemical shifts were first predicted and then the spectrum of 27 obtained. The good correlation between predicted and observed values merited inclusion of 27 as an example of the approach to use in assigning the

chemical shifts of large molecules using model compounds.

The assignment of the chemical shifts of perylene (24) is simplified due to the symmetry which greatly reduces the number of resonances. For the protonated carbons, only three resonances will be observed. The resonance at $\delta=120.15$ is assigned to C-1,6,7,12. In the ^1H NMR spectra the protons attached to this position give a lowfield doublet at $\delta=8.14$ which is well removed from the other multiplets and easily allows the use of selective decoupling technique for assignment. As would be predicted on the basis of C-4,5 of phenanthrene (8), C-1,6,7,12 of 24 is the peak furthest upfield. The proton NMR spectrum has been assigned (13) for 24 in CS_2 and that in CDCl_3 is very similar. The results of this study assigned H-2,5,8,11 and H-3,4,9,10 to $\delta=7.38$ and $\delta=7.57$, respectively. Using this, the proton selective decoupling allowed $\delta=126.48$ and $\delta=127.78$ to be assigned to C-2,5,8,11 and C-3,4,9,10, respectively. One might have predicted C-2,5,8,11 to come at $\delta=126.50$ on the basis of C-3,6 of 8, and C-3,4,9,10 to be like C-1,4,5,8 ($\delta=128.34$) of 1. Both are very near the observed results.

The quaternary carbons of perylene (24) are assigned using intensity differences and model compounds. Two of these three resonances are in a 2:1 intensity

ratio and so $\delta=131.24$ and $\delta=134.75$ are assigned to C-6a,6b,12a,12b and C-3a,9a, respectively. Alternatively, C-4a,4b of phenanthrene (8) could be used to predict the C-6a,6b,12a,12b, while C-4a,8a of naphthalene 1 could be used to predict C-3a,9a. The remaining resonance, $\delta=128.73$ is assigned to C-9b,12c on the basis of its very low intensity relative to that of the C-3a,9a resonance. This is expected since they are far removed from any protons on the periphery and thus have a very long T_1 . This absorption was of such low intensity that a spectrum of a solution of 24 and $\text{Cr}(\text{acac})_3$ was needed to verify its reality.

While 1,12-benzoperylene (25) also possesses an axis of symmetry, the assignments are not made as simply as in 24. The analysis of the ^1H NMR spectrum has not been published to date and only the lowest-field doublet at $\delta=8.95$ can be assigned by inspection to H-7,8. It is far enough removed from the remainder of the complex multiplet to permit selective proton decoupling. On this basis, $\delta=120.60$ is assigned to C-7,8, which also could have been made using C-1,6,7,12 of 24 as a model. There is only one double intensity peak in the spectrum of 25 at $\delta=127.30$, which must be assigned to C-3,4,11,12. These carbons should be very much like C-4,5,9,10 of pyrene 21 ($\delta=127.50$); this is most easily envisioned by using the axis of symmetry to observe only one half of 25, a

pyrene-like structure. The absorption at $\delta=125.53$ is assigned to C-5,10 by using C-1,3,6,8 of pyrene (21) as a model ($\delta=125.06$). The remaining two protonated carbon resonances for 25, $\delta=126.42$ and $\delta=126.12$, are assigned to C-1,2 and C-6,9 for which C-5,8 of perylene (24) or C-3,6 of 8 serve as models.

The quaternary carbons of 1,12-benzoperylene (25) can, on consideration of intensities alone, be separated into two groups: those on the periphery and those on the interior. For the latter type, use of $\text{Cr}(\text{acac})_3$ was necessary to observe the resonances to be at $\delta=125.61$ and $\delta=123.83$. These are centered about $\delta=124.84$, the value predicted for internal quaternary carbons using C-10b,10c in pyrene (21). The C-9b,12c resonance of perylene (24), $\delta=128.73$, is used as a starting point for specifically assigning C-4b,12c of 25. To approximate the effect of the ring addition at this position, the rings defined by C-2a,3,4,4a,5,6,7,7a,4b,4c are considered as a naphthalene moiety with C-4b in the quaternary position. The ring addition to the perylene base has the effect of a ring added to the naphthalene moiety to make phenanthrene. Using C-4a,4b of 8 and C-4a,8a of 1, the effect on C-4b in the perylene base would be a 3.2 ppm upfield shift. The predicted chemical shift of 125.5 ppm for C-4b,12c in 25 agrees well with the observed value of $\delta=125.61$. The

other internal quaternary carbon, C-4c,12b, is assigned to the remaining peak at $\delta=123.83$.

The C-2a,12a resonance of 25 is best approximated with C-8a,10a of phenanthrene (8), and C-7a,7b of 25 should be like C-4a,4b of 8 (the overall shape of 25 is like two phenanthrene molecules joined together). Thus, the resonances at $\delta=132.17$ and $\delta=130.34$ are assigned to C-2a,12b and C-7a,7b, respectively. The remaining peak at $\delta=129.13$ is assigned to C-4a,10a of 25. The observed intensities of these three quaternary carbon resonances verify the results obtained from the model compounds. The C-7a,7b carbons have only one adjacent protonated carbon and therefore their resonance intensity should be lower than those of the other two, which should be equal since all have two adjacent protonated carbons. This is in fact observed.

Anthanthrene (26), as already mentioned, displayed resonances for only its protonated carbons due to its very low solubility in CDCl_3 -- despite a favorable symmetry and use of $\text{Cr}(\text{acac})_3$, the quaternary carbon resonances were too weak to observe. The ^1H NMR of 26 has not been totally analysed in the literature, but it is simple to assign the lowest field singlet, $\delta=8.75$, integration of two, to H-6,12. This is in agreement with a value of $\delta=8.67$ which has been predicted by a theoretical study (59). Use of the experimental value in a selective

proton decoupling experiment allows $\delta=124.16$ to be assigned to C-6,12. For C-4,10 and C-5,11 of 26 the resonances should come at lower field than those of any other carbon. In benzo[a]pyrene C-4 and C-5 come near $\delta=127.5$ and the effect of adding the sixth ring to this nucleus is best determined by considering a naphthalene to phenanthrene network for its transmission (just as in calculating the chemical shift of C-4b,12c in 25). Defining the naphthalene system with C-3a,4,5,5a,12c,11b,-11a,12,12a,12b and using C-2,3 in 1 and 8, the effect of another ring should be a small downfield shift of about 0.7 ppm and thus C-4,10 and C-5,11 are expected to have chemical shifts near $\delta=127.99$ and $\delta=128.32$, respectively*. For C-1,7, C-2,8 and C-3,9 of 26, the chemical shifts of C-1, C-2 and C-3 of benzo[a]pyrene (22) can be used as a starting point. The addition of the sixth ring to the benzo[a]pyrene nucleus and its effect on the three model positions is estimated by comparing these same carbons in anthracene (5) and naphthalene (1).

* Alternatively, the naphthalene system could have been defined by C-12b,3a,4,5,5a,6,6a,11c,11b,12c. For C-4,10 of 26, C-2 of 1 and 8, and for C-5,11 of 26, C-1 of 1 and 8 could have been used to determine the effect of the ring addition onto 22 to give 26. Both approaches give the same answer.

Thus, the predicted chemical shifts in 26 are: $\delta=125.03$ for C-1,7, $\delta=125.18$ for C-2,8, and $\delta=124.08$ for C-3,9. The actual values observed are: for C-1,7 and C-2,8, $\delta=125.85$ and $\delta=125.89$ (cannot be unambiguously assigned), and for C-3,9, $\delta=123.87$.

The assignments for the last aromatic compound, dibenzo[a,e]pyrene (27), can only be made by use of model compounds. Its low solubility in CDCl_3 has made it impossible to attempt a selective proton decoupling experiment, as well as eliminating the observation of any of the quaternary carbons. The molecule itself can be viewed as having a dibenz[a,c]anthracene (12) or a benzo[a]pyrene (22) nucleus. One of these two compounds provide the basis for the chemical shift of each carbon in 27, to which must be added the effect of the addition of the last ring. This ring effect can be calculated from a number of model systems depending on which carbon in 27 is being considered. Some of these are naphthalene (1) going to anthracene (5), anthracene (5) going to benz[a]anthracene (14), and benz[a]anthracene (14) going to dibenz[a,c]anthracene (12). Table 12 is a simplified presentation of the assignment techniques for each carbon in 27.

Table 12. Prediction of the ^{13}C Chemical Shifts of dibenzo[a,e]pyrene, 27.

Carbon in 27 to be assigned	Model compound from which basic chemical shift, δ_M is taken		Model system used to calculate the effect of the ring addition, $\Delta\delta$						Observed Chemical Shift in 27			
	Cpd.	Carbon position	δ_M	Compound #1 Carbon position	δ_A	Cpd. Carbon position	Compound #2 Carbon position	Effect of ring addition $\Delta\delta = \delta_B - \delta_A$		Predicted chemical shift in 27 $\delta_M + \Delta\delta$		
C-12	22	C-10	122.87	5	C-1,4,5,8	128.34	14	C-8	128.32	-0.02	122.85	122.82
C-13	22	C-11	122.00	1	C-2,3,6,7	125.84	5	C-2,3,6,7	125.24	-0.60	121.40	121.89
C-3	12	C-4	123.66	14	C-4	128.49	22	C-3	124.68	-3.81	119.85	120.25
C-8	12	C-9	122.03	14	C-7	127.21	22	C-6	125.38	-1.83	120.20	120.51
C-4	12	C-5	123.66	1	C-1,4,5,8	127.92	5	C-1,4,5,8	128.34	+0.42	124.08	123.84(a)
C-7	12	C-8	123.41	1	C-1,4,5,8	127.92	5	C-1,4,5,8	128.34	+0.42	123.83	123.66(a)
C-1	22	C-1	124.61	14	C-2	126.71	12	C-2	127.57	+0.86	125.47	126.11
C-2	22	C-2	125.78	14	C-3	126.65	12	C-3	127.44	+0.79	126.57	126.64
C-14	22	C-12	128.00	1	C-2,3,6,7	125.84	5	C-2,3,6,7	125.24	-0.60	127.40	127.87
C-5	12	C-6	127.44	1	C-2,3,6,7	125.84	5	C-2,3,6,7	125.24	-0.60	126.84	127.55(b)
C-6	12	C-7	127.57	1	C-2,3,6,7	125.84	5	C-2,3,6,7	125.24	-0.60	126.97	127.75(b)
C-9	22	C-7	128.77	14	C-8	128.32	12	C-10	128.09	-0.23	128.54	129.11
C-10	22	C-8	125.88	14	C-9	125.67	12	C-11	126.04	+0.37	126.25	126.25
C-11	22	C-9	125.91	14	C-10	125.56	12	C-12	126.04	+0.48	126.39	126.25

(a), (b) denote possible interchange of assignments.

The first six carbons predicted in 27 are all those expected to resonate at high field. These protonated carbons are involved in a bay region and it has been consistently observed in these situations that the steric interactions of the protons on these carbons result in upfield shifts for the carbons such that $\delta < 124$. For dibenzo[a,e]pyrene (27) the spectrum clearly shows six resonances with $\delta < 124$ and the remainder with $\delta > 126$. Of these resonances with $\delta < 124$, the agreement between observed and predicted chemical shifts is quite good. For C-13, C-14, C-5, and C-6, the effect of addition of the last ring to the reference compound is -0.60 ppm. For all of these however, the observed chemical shifts are, on the average, 0.6 ppm greater than those predicted. Thus, it would seem that the calculated effect is unnecessary and that the model compound alone predicts the chemical shift quite well because these positions are isolated from the effect of the ring addition. The one double intensity resonance in the spectrum of 27, $\delta = 126.25$, is predicted to be that of C-10 and C-11.

K. Assignment of the ^{13}C Chemical Shifts of Methyl Groups in 1-27. The Results for Table 11.

The assignment of the substituent methyl groups in the various compounds studied is for the most part simple.

All but one of these compounds display only a single methyl resonance, and, since they occur in a characteristic region far removed from the aromatic region, their identity is quickly established. For the methyl's of 3, 4, 16 and 19, the chemical shifts are those reported by Wilson and Stothers (35).

For 9-methylanthracene (7) and 9-10-dimethylanthracene (6), the observed shifts are very similar, being $\delta=13.83$ and $\delta=14.02$ respectively. The small downfield shift of the signal of the para-oriented methyls in 6 relative to that of the methyl in 7 mirrors the observed shift in 3 and 4.

As expected, the methyl carbon of 7-methylbenz[a]anthracene (15), $\delta=14.09$, is observed to be very close to that of the methyl of 7. For 7,12-dimethylbenz[a]anthracene 17, the methyl carbon attached to C-7 is expected to be similar to that of 15. However, as in 3 and 4 and in 6 and 7, its position should be shifted downfield by 0.1 to 0.2 ppm due to the second methyl. The observation of the resonance at $\delta=14.16$ in 17 confirms these expectations. The methyl attached to C-12 in 17 is thus assigned to $\delta=20.96$. The 6.80 ppm downfield shift of this methyl carbon relative to that at C-7 is due to the strong δ steric interaction between the C-12 methyl group and C-1. The same effect has already been noted in 3 and 16.

and has been used to determine that the chemical shift of C-1 in 17 is shifted 6.61 ppm downfield relative to C-1 in 15.

For 3-methylcholanthrene, the methyl resonance is assigned to $\delta=18.65$ on the basis of the uniqueness of the chemical shift. This value is ca. 3 ppm upfield from the methyl in 2-methylnaphthalene (19) and is likely due to a γ interaction with the C-2 methylene function.

L. Summary

In this chapter the ^{13}C chemical shifts have been presented, Tables 3-11, and the details of each assignment given for various polynuclear aromatic hydrocarbons. The study began with the simplest two-ring system, naphthalene (1), and progressed to compounds having six condensed aromatic rings. In addition, various simple but carcinogenically-important derivatives of these compounds have been included. For all compounds, the observed chemical shifts, most of which fall within a very narrow range of about 12 ppm, have been successfully assigned using a variety of techniques.

There are chemical shift trends which can be noted in these results for the polycyclic aromatic compounds. In general, there are a number of factors contributing to the chemical shift of each carbon. This

makes it difficult to identify and separate the various individual components. There are a few cases, however, where a clear empirical trend has developed.

- (i) For an angular arrangement of three aromatic rings, the protonated carbons involved in a γ steric interaction in the bay region, such as C-1 and C-8 of phenanthrene (8), will be found with $\delta < 124$.
- (ii) For an angular arrangement of aromatic rings where there is a strong δ steric interaction between two protonated carbons, there will be a large downfield shift of 5-7 ppm. In the current study this interaction was only observed in 7,12-dimethylbenz[a]anthracene, (17). The effect was ca. 6.6 ppm for both the aromatic carbon, C-1, and the saturated methyl carbon. This large δ downfield shift is due to the strong steric interaction between the proton at C-1 and the methyl group substituted onto C-12. In most cases the effect on the δ carbon from the point of substitution is negligible (< 1 ppm). Furthermore, it is expected that the steric δ effect would be similar to the steric γ effect and lead to an upfield shift (as explained either by the

popular theory of the induced C-H bond polarization by the non-bonded $H\cdots H$ interactions (60), or by these forces and the nature and conformational relationship of the bonding connecting the interacting groups (61)). However the δ steric effect observed for C-1 of 17 is neither small nor of an upfield nature. This 6.6 ppm downfield δ shift is one of the largest observed and is one of the few examples of a strong δ steric shift in an aromatic compound. The rigid conformation of the bay region of 7,12-dimethylbenz[a]anthracene causes C-1 and the methyl at C-12 to retain very close contact and thus leads to the large observed effect. The simplest polynuclear aromatic hydrocarbon where one would expect such an effect is benzo[c]phenanthrene.

The ^{13}C spectrum for this compound has been assigned (44) and the C-1 and C-12 involved in the δ steric interaction are found to be shifted 5.3 ppm downfield relative to C-1,8 in phenanthrene (8).

- (iii) For unsubstituted polycyclic benzenoid aromatic ring systems, quaternary carbons on the periphery of the molecule are generally found with $\delta > 128.7$. Quaternary carbons which are internal have a $\delta < 128.7$. In addition, resonances of these internal carbons have a very long T_1 and thus a greatly attenuated intensity relative to those of the outer carbons when the spectrum is rapidly accumulated.

These relationships have been obtained from a rather limited number of compounds. They do, however, lead to readily recognizable spectral features and thus merit further investigation with other compounds. The remaining carbons in the compounds studied do not partake in such strong steric interactions but rather undergo smaller changes due to long-range effects. These cannot be generalized in the same manner as the above.

Chapter 4

CONCLUSIONS

A. Methods of Assigning the ^{13}C Chemical Shifts of Polynuclear Aromatic Hydrocarbons

The objective of this study was to assign the ^{13}C chemical shifts of an important class of carcinogens, the polycyclic aromatic hydrocarbons and some of their important derivatives. Due to the very narrow chemical shift range a number of different techniques were invoked or developed to assign these chemical shifts as unambiguously as possible.

One valuable method used in the current work was that of single frequency selective proton decoupling. This technique is important because it gives unambiguous assignments, and it only requires an assigned ^1H spectrum in order to be employed. Fortunately, many of the polynuclear aromatic hydrocarbons in this current study have had their proton spectrum completely analyzed and are thus amenable to this technique. However, there are problems associated with selective decoupling which limit its use to only a limited number of carbons of each compound. Since so many of these ^1H spectral resonance multiplets are very close to one another it is not possible, for many positions, to decouple one without decoupling others. In addition,

the fact that the many ^{13}C resonances are in a small spectral region sometimes makes it difficult to identify readily the one being selectively decoupled. It becomes necessary to move the decoupler offset about and observe many ^{13}C spectra in order to identify the one resonance undergoing decoupling. While it might be possible to overcome these problems by going to a higher Larmor frequency, there is still the problem that many of these compounds give a spectrum with poor signal-to-noise. It would be impractical in such cases to obtain many spectra in order to assign one carbon. A further complication for the technique is that it is not applicable to the assignment of quaternary carbons. Thus, selective decoupling will play a limited, but important, role in the assignment of ^{13}C spectra of polynuclear aromatic hydrocarbons.

A second technique for unambiguously assigning the ^{13}C resonances of polynuclear aromatic hydrocarbons is that of selective deuteration. A very serious drawback to this method is that it requires an expertise in synthesis to introduce a ^2H atom at a predetermined position. This is further complicated by the relative inertness of the polycyclic aromatic hydrocarbons. However, the technique has many advantages. Firstly, one spectrum is all that is needed to observe the changes it brings about. Secondly, the spectral changes are very dramatic for the deuterated

carbon; it can be assigned immediately upon comparing the spectra of the compound and its deuterated analogue.

Thirdly, information can be obtained not only for the site of deuteration, but also isotope shifts are observed for carbons β and γ to that position. This last point is very important because it is a means of assigning quaternary carbons. Even in more complex molecules these small isotope shifts would be useful if they only separated the quaternary carbon resonances into two types, either those β or γ to the site of substitution or those further removed. Thus, selective deuteration is a very important technique for assigning the ^{13}C spectra of polynuclear aromatic hydrocarbons. It is important to note though, that the use of the ^{13}C -D coupling in these deuterated compounds cannot be used as a reliable means of making the assignments of the quaternary carbons. As shown in the deuterated derivatives (9 and 10) of phenanthrene (8), the vicinal couplings, $^3J_{13}\text{CCCD}$, are sensitive not only to the dihedral angle but also to the pathway.

Due to the difficulty in assigning the resonances of the quaternary carbons, one new technique was developed to aid in this process. The property of the quaternary carbons of the polynuclear aromatic hydrocarbons which is important to this method is their spin-lattice relaxation time, T_1 , and its dependence on the number of

adjacent protonated carbons. The relationship which exists is that the T_1 of a carbon increases with the sixth power of the distance between it and the neighboring hydrogen atoms. Thus, the observed T_1 value for a protonated carbon will be substantially less than that of a quaternary carbon. For ^{13}C spectra accumulated with a relatively fast pulse repetition rate, this results in a greatly reduced resonance intensity for quaternary carbons since their distance to the nearest hydrogen is much greater than for a carbon with a directly-bonded hydrogen. This feature readily identifies most nonprotonated carbons in the ^{13}C spectra.

If one is able to measure accurately the T_1 values it should be possible to extend these arguments and use the T_1 values to identify further and assign the quaternary carbons of these polycyclic aromatics. For these compounds, the quaternary carbons have either none, one or two adjacent carbons with directly-bonded hydrogen atoms (depending on the type of ring junction). The dipolar relaxation times of these three situations will decrease as one goes from none to two adjacent carbons. The one assumption necessary to validate this argument is that of equal τ_C values for all carbons. For the polynuclear aromatic hydrocarbons this is not a bad assumption and it can only be complicated by anisotropic rotation. While there are other mechanisms contributing to the relaxation

of these quaternary carbons (as indicated by their partial nuclear Overhauser enhancement), the contribution from the dipole-dipole mechanism is sufficient for the observed T_1 's to reflect the differences due to the number of adjacent protonated carbons. This was demonstrated for the quaternary carbons of four compounds, phenanthrene (8) and its two deuterated derivatives 9 and 10, and for dibenz[a,c]-anthracene (12).

The complications which prevent this method from being applied to all these aromatic compounds are: the need of good S/N for accurate measurements, long experimental times needed to measure these very long T_1 values, and the low solubility and availability of some compounds. Thus, the T_1 experiments become difficult to do, and the results may lack the precision to merit use as an assignment technique. The method adopted here was based on the observation of the peak intensities of the quaternary carbons of these compounds. Under rapid pulse repetition rates used to obtain the spectra, a difference in the quaternary carbon intensities was noted, and the number of peaks with the same intensity corresponded to the number of quaternary carbons with a different number of adjacent protonated carbons. It would be expected that the longer the T_1 , the smaller should be the intensity. This relationship was substantiated with the four compounds for which quater-

nary carbon T_1 values had been determined. The reliability of this method depends on the peak intensities differing by an amount greater than the spectral noise and on all quaternary carbon resonances having the same line width. Since these problems were not encountered in the spectra where quaternary carbons were visible, and since the compounds (with a few exceptions like 3-methylcholanthrene (20)) belong to a homologous series, the peak intensities were used to indicate the T_1 of the carbon. These in turn were related to molecular environments and assignments were made. Ideally, the T_1 values would be determined to establish a greater degree of faith for these quaternary carbon assignments. Since this is impractical except for a few cases (which validate the method for the series), the assignments based on peak intensity were used in conjunction with those made from model compounds. This method should be applicable to all polycyclic aromatic hydrocarbons.

Since all of the three previously mentioned techniques have difficulties or assumptions associated with them, it became necessary to adopt the approach taken in this study. The assignments were undertaken beginning with the simple molecules for which deuterium isotope effects or selective proton decoupling was possible. Having unambiguous ^{13}C chemical shifts it became possible to use this as a model compound and progress to a more com-

plicated one for which the model was applicable. For the methylated derivatives of some compounds, simpler methylated model compounds were used in conjunction with the parent compound to complete the assignments. In this way a "pyramid" of assignments was made, those of the current compound depending on those of the preceding compound. To make this credible and limit the propagation of any error, it was possible to obtain a few isolated cases where either deuteration or a selective proton decoupling was practicable. These allowed for a check on the approach to the assignments using model compounds. In all cases these unambiguous techniques verified the chemical shift obtained from the model compound.

With the use of model compounds to predict chemical shifts, a practical point has arisen with regards to the polycyclic aromatic compounds. It has been found that when a carbon in two compounds is used to predict the effect of some molecular perturbation, the best results between observed and predicted are achieved when the carbon in the two compounds is of the same type. That is, it is either a protonated carbon or a quaternary carbon in both molecules. When it is one type in one compound and the other type in the second compound, the results are often meaningless.

Thus, for all the carbons of these compounds,

the most extensively used technique was that of chemical shift trends in model compounds. Fortunately other unambiguous techniques were available to add credibility to the assignments made in this way. The final result is a self-consistent set of ^{13}C chemical shifts in which it is possible to express a reasonable degree of confidence.

B. Correlating ^{13}C Chemical Shifts and Carcinogenic Activity of Polynuclear Aromatic Hydrocarbons

The ^{13}C chemical shift data of this series of carcinogenic polynuclear aromatic hydrocarbons and important derivatives represent direct experimental information on the molecular skeleton of the molecules, the carbon atoms. Unlike the complicated ^1H NMR spectra in which hydrogen atoms are observed (and thus indirectly only the protonated carbons), the ^{13}C NMR spectrum gives one resonance per carbon except in the case of symmetry or exceptional similarity in molecular environments for different carbons. Each part of the molecule can be monitored and local properties should in principle be assessable from the ^{13}C chemical shift of each carbon. Ideally, one wishes to have an intrinsic molecular property which can be experimentally measured and which correlates with a carcinogenic mode of reactivity. In terms of the K-L theory for the

carcinogenic reactivity of these compounds, the ^{13}C chemical shifts should be of interest. This theory is based on the electronic properties of the individual carbons, bonds or regions, which in turn infer particular modes of reactivity, some of which lead to carcinogenesis. The problem becomes one of trying to relate ^{13}C chemical shifts with an electronic property and thus the corresponding reactivities.

The complication which arises is that the ^{13}C chemical shift value for a particular carbon is composed of many factors, only one of which is the electron density. There are at present no simple methods of separating a ^{13}C chemical shift into its different components (33,62).

Due to this problem one can only make a first approximation that the ^{13}C chemical shift value reflects the electron density of a carbon. This quickly leads to difficulties. For example, consider the results shown in Figure 10 where the charge density and the ^{13}C chemical shifts are given for three of the compounds from the current study. The charge density used here is calculated as the difference between the nuclear charge ($Z_A=6$ for carbon) and the gross atomic charge (i.e. electron density) from a simulated ab initio molecular orbital calculation (63). As is readily apparent, there is no correlation between the electron densities and the ^{13}C chemical shifts for the

polynuclear aromatic hydrocarbons. Therefore, one is faced with having to derive the components of the ^{13}C chemical shifts in order to observe such a correlation. It can also be noted that the charge densities (and therefore the electron densities) and the chemical shifts are the same for the K-region of each molecule, despite their widely different reactivity noted in the K-L theory. This is not surprising since the K-region was defined on the basis of the π electrons isolated at the bond and not the total electron density of two carbons between which the bond exists. In order to compare the ^{13}C chemical shifts with this π electron property it would be necessary to carry out further calculations to determine a parameter which is related to the π electron contribution to the chemical shift of each carbon. Having obtained these values for each carbon atom, it would still be necessary to relate them to the nature and the reactivity of the bond between the two atoms.

Therefore, if one wishes to use the ^{13}C chemical shifts to provide information on the electronic properties and reactivities (and thus the carcinogenic potency) of the polynuclear aromatic hydrocarbons, it would be necessary to carry out much more sophisticated calculations. These more detailed calculations go beyond the scope of this thesis.

C. Suggestions for Future Work

It is possible that these data may be of further use. The following is a list of suggestions for future work.

The compounds for which the ^{13}C chemical shifts have been obtained and assigned do not constitute the complete aromatic hydrocarbon series. There are other possible geometric arrangements of the cyclic rings, many of which are commercially available and carcinogenic. These data would be of interest to, and a foundation for, ^{13}C NMR studies of derivatives or related compounds. As one progresses into the larger hexacyclic systems, such as the dibenzopyrene series (among which are potent carcinogens), limited solubility in CDCl_3 becomes a problem. It may be possible to use $\text{Cr}(\text{acac})_3$ in CDCl_3 or with a mixed solvent system to observe all the resonances. Increased solubility might also be found with the potent solvent hexafluoroacetone. The use of liquid crystal solvents presents itself as another solution. Many of the liquid crystals are composed of molecules containing aromatic rings, and thus they may solvate these larger molecules. The problem is also aided by the fact that current commercial spectrometers have improved sensitivity. Having a greater collection of ^{13}C chemical shifts might

allow removal of the ambiguity of some assignments for the current compounds and also check the correctness and consistency of all of them. Since these compounds also are a very homologous series, it may be possible to determine an empirical relationship for predicting their ^{13}C chemical shifts just as the Lindeman-Adams rules (64) allow prediction of the ^{13}C chemical shifts of alkanes.

With a theoretical approach there is the possibility of separating the ^{13}C chemical shifts into various components which reflect the different forces at each atom. One of the components, such as ring current effects for example, may correlate with an enhanced reactivity for a particular region and thus lead to new understanding of an intrinsic property which defines carcinogenic activity. If this were possible, it might also be possible to use the chemical shifts for screening other polynuclear aromatic hydrocarbons for carcinogenic activity. This theoretical approach to carcinogenesis also has applications in the next suggestion.

Due to the predominance of hydroxylated compounds found in the original metabolic studies of the aromatic hydrocarbons, the ultimate carcinogen was thought to be a K-region epoxide which would bind to DNA and lead to the genetic disruption of cellular order and cancer. Much work was put into synthesizing, biological testing

and searching for such compounds (see (4) for examples). With the introduction of sophisticated high pressure liquid chromatography and spectroscopic identification methods, different hydroxylated metabolites have been isolated from in vitro metabolism of the compounds with microsomal systems. When these compounds were synthesized and tested for carcinogenic activity, they were more potent than the parent compound. The K-region epoxides, on the other hand, were rarely ever more potent than the parent. Thus, since 1972 a new concept of the ultimate carcinogen has arisen. In it the K-region is no longer thought to play the central role in the cancer initiation reaction.

These studies have most extensively been carried out on the potent carcinogen, benzo[a]pyrene (BP). In 1974, Sims concluded that the ultimate carcinogens were the diastereomeric isomers of 7,8-dihydro-7,8-dihydroxy-benzo[a]pyrene-9,10-oxide (65). The evidence comes from

numerous investigations and includes* :

- (i) the observation that liver microsomes metabolize BP-7,8-dihydrodiol to products that bind more extensively to DNA than do the products of the metabolism of BP itself (66).
- (ii) the observation that the nucleoside adducts formed in vitro between an epoxide of BP-7,8-dihydrodiol and DNA are similar to those adducts obtained from cells in culture that had been exposed to BP (65,67).
- (iii) the observation that the same DNA nucleoside adducts are formed when BP is applied to mouse skin as when the dihydrodiol epoxide is metabolized by microsomal preparations in the presence of DNA (68).
- (iv) the spectroscopic evidence, including that from ^1H NMR, of the in vitro and in vivo binding of the two isomers to the various nucleic acids of RNA and DNA. Since the binding is especially strong to guanosine, detailed studies were carried out on this adduct and the complete structure was found to be as follows (69-71).

*In this section only key references are given because of the large number of papers which have appeared.

This is an area of very intensive research and there are numerous papers on all aspects of it (see (72) as an example of one review of the literature).

With recent developments of microprobes and increased sensitivity for ^{13}C NMR spectrometers, it now becomes possible to obtain natural abundance spectra from 400-1000 μg of material. Thus, it is feasible to use ^{13}C NMR for identification of the various products formed when the aromatic hydrocarbons are metabolized. It has the tremendous advantage of displaying each carbon atom individually and one should be able to observe readily the changes which occur in all regions of the molecule. As mentioned, benzo[a]pyrene has been most extensively studied to date, but the same types of experiments are now being reported for other compounds. These include: 7-methylbenz[a]anthracene (73,74), benz[a]anthracene (75,76), 7,12-dimethylbenz[a]anthracene (77-79), and 3-methylcholanthrene (80,81). In two of these reports, (77) and (78), ^1H NMR has been cited as one of the techniques used to identify the adduct formed between the carcinogen and the nucleic acid. The possibilities of using ^{13}C NMR in such studies would be greatly enhanced if ^{13}C labelling of various components could be achieved. The clarity of the ^{13}C NMR information would make it a valuable tool in these metabolic studies.

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PART II

THE HEMIACETAL INTERMEDIATE OF THE OXIDATIVE
DEETHYLATION METABOLISM OF PHENACETIN IN RAT
LIVER MICROSOMES

Chapter 1

THE METABOLISM OF PHENACETIN

A. Introduction

Phenacetin (p-ethoxyacetanilide) has been

extensively used as a pharmaceutical drug. It was introduced in 1887 as a less-toxic derivative of and substitute for acetanilide. Phenacetin has proven analgesic (alleviation of pain) and antipyretic (prevention of fever) properties. Its most common usage has been in combination with caffeine and acetylsalicylic acid (ASA) in analgesic cold tablets (1).

Since the 1950's there has been considerable work on establishing the association between chronic abuse of these analgesics and damage of the functional units of the kidneys, the renal tubules. This so-called analgesic nephropathy has been extensively reviewed (2-4). Since phenacetin was suspected of being the nephrotoxic component, it was removed from many preparations and since 1964 cannot be included in a prescription without a warning label.

There has been recent evidence (5,6) that phenacetin causes hepatic necrosis* in experimental animals.

Further studies have implicated phenacetin abuse with pathogenesis of the interstitial connective tissue and with renal pelvic tumors (7-9).

This documented toxicity of phenacetin has led to numerous studies on its metabolism, a review of which can be found in (10). The aim of such studies has been to gain a molecular level understanding of the toxic action of this drug. This portion of the thesis will deal with one important aspect of the metabolism of phenacetin.

B. Kinetic Study of the Oxidative Deethylation of Phenacetin and Toxicity Tests

One of the first steps in phenacetin metabolism is an oxidative deethylation to form acetaminophen (p-hydroxyacetanilide), as shown in Figure 1. This process occurs primarily as a result of the action of the hepatic (liver) microsomal enzymes; 75 to 80% of administered phenacetin rapidly undergoes this fate (1). The microsomal oxygenase enzymes require Mg^{+2} as a cofactor, and are

* Hepatic necrosis is defined as the irreversible death of localized areas of the liver, usually as individual cells, groups of cells or tissue, but sometimes the entire organ.

Figure 1. The proposed mechanism for the oxidative deethylation of phenacetin by hepatic microsomal enzymes.

dependent on the presence of reduced nicotinamide adenine dinucleotide phosphate, NADPH, for the original electron for the P-450 electron transport system which will ultimately hydroxylate the phenacetin using O_2 . Different studies have established this metabolic route in several mammals: man (11), rat (12), cat (13), rabbit (12, 14), guinea pig (12) and dog (13, 14).

The generally accepted mechanism of such a deethylation (15) would predict that the methylene carbon would be first oxidized to a hemiacetal intermediate (see Figure 1), followed by release of acetaminophen and acetaldehyde. A similar mechanism, as shown in Figure 2.A, has been proposed by Renson et al. (16) for the demethylation of p-methoxyacetanilide; oxidation of the methoxy carbon and not a methoxy displacement process. The proposed hemiacetal intermediate would also be analogous to the intermediate carbinolamine believed formed when a secondary or tertiary amine undergoes metabolic N-dealkylation (17-20), as shown in Figure 2.B.

The first evidence, albeit indirect, for the existence of the hemiacetal intermediate in phenacetin metabolism came from a kinetic study by Garland et al. (21). The results reported were for metabolism in rabbit liver microsomes, and were based on a gas chromatograph-mass spectrometer method to analyze for phenacetin and acetamidophen (22). They determined the individual rate,

Figure 2. Metabolic processes believed to have an intermediate analogous to the hemiacetal intermediate in phenacetin metabolism.

A. Demethylation of p-methoxy acetanilide (16).

B. Dealkylation of secondary and/or tertiary amines (17-20).

k , for each phenacetin concentration and the reaction's Michaelis-Menton K_m and V_{max} constants.

The study involved the use of phenacetin and two of its specifically deuterated derivatives, as shown in Table 1. Included in this table are the results from the kinetic studies using all three phenacetin compounds. The deethylation reaction rates, k , for the three were determined at five different concentrations. The kinetic deuterium isotope effect, defined as the ratio of the deethylation rate of phenacetin to that of the deuterated derivative, k_H/k_{D_2H} , was determined for the two deuterated phenacetins by taking the average of their respective five values of this ratio (one for each of the concentrations). The calculated kinetic deuterium isotope effect for each compound is contained in Table 1.

Kinetic theory predicts that if the rate-determining step of a reaction occurs with cleavage of a bond with a deuterium atom substituted for a hydrogen, there should be a substantial decrease in rate. Such a substitution at the bond being cleaved leads to a primary kinetic isotope effect which theory predicts to be $k_H/k_{D_2H} > 1.44$. The phenacetin derivative with the two deuterium atoms on the methylene carbon of the ethyl group gave a value of 1.6. and was classified as exhibiting a primary deuterium isotope effect. The phenacetin analogue with three deuterium atoms on the methyl carbon of the ethyl

Table 1.

The kinetic data for the metabolism of phenacetin and its deuterated derivatives in rabbit liver microsomes. The data were obtained from 10 minute incubations of each compound (at 12.5, 16.6, 25, 50 and 100 μ M concentrations) with the microsomes (21).

Compound	K _m (μ M)	V _{max} (nmoles/mg protein/min)	Kinetic Deuterium Isotope Effect (k _H /k ₂ H)	Interpretation of Deuterium Isotope Effect
$\text{CH}_3\text{-CH}_2\text{-O-}$ Phenacetin	55.6 $\pm$ 2.1	7.55 $\pm$ 0.31	-	-
$\text{CH}_3\text{-C}^2\text{H}_2\text{-O-}$ p-[1,1- ² H]Ethoxy- acetanilide	55.8 $\pm$ 5.2	4.29 $\pm$ 0.49	1.61 $\pm$ 0.19	primary (hemiacetal formation rate determining step)
$\text{C}^2\text{H}_3\text{-CH}_2\text{-O-}$ p-[2,2,2- ² H]Ethoxy- acetanilide	49.6 $\pm$ 2.2	7.33 $\pm$ 0.35	1.03 $\pm$ 0.09	β -secondary (free radical or S _N 2 mechanism)

group yielded a ratio of 1.03, a β -secondary isotope effect:

On the basis of these results, Garland et al. concluded that the data were consistent with, and in-direct proof for, the formation of the hemiacetal intermediate, a process requiring the cleavage of a bond between the methylene carbon and one of its hydrogens. The primary isotope effect is smaller than, although similar to, that noted for the O-demethylation of o -[1,1,1- 2 H]methoxynitrobenzene (average $k_H/k_{^2H} = 1.98$) (23) and for the O-deethylation of p -[1,1- 2 H]ethoxynitrobenzene ($k_H/k_{^2H} = 3$) (24). The lack of any β -secondary deuterium isotope effect for the phenacetin metabolism was not readily interpreted by Garland et al., since in other drugs, effects of ca. 1.50 had been observed and interpreted in terms of considerable sp^2 character in the transition state. The few published studies which are relevant to the β -deuterium effect (and its magnitude) observed by Garland for phenacetin suggested either a free radical or $Sn2$ reaction mechanism.

In addition to these kinetic studies Garland has arranged for a toxicity test of phenacetin and its two deuterated analogues (25). Using hamsters, the relative hepatotoxicities of the three compounds were determined. While phenacetin and the p -[2,2,2- 2 H]ethoxyacetanilide displayed much the same potency, the p -[1,1- 2 H]ethoxyacetanilide displayed a reduced hepatotoxicity.

This evidence strongly implicated the deethylation reaction with the toxic property of phenacetin. Further, if the hemiacetal is actually one of the metabolites it may be the ultimate toxic species. Being an unstable molecule, it is not reasonable that it may be an initiator in a chain of reactions which lead eventually to the toxic effects of phenacetin.

C. Scope of the Research Project

With these references to the key role of the deethylation reaction, and especially to the hemiacetal intermediate, it became important to obtain direct evidence for the hemiacetal's existence. This had not been possible for Garland using gas chromatography-mass spectrometry due to the instability of the hemiacetal and the harsh conditions of the technique.

However, during the course of a study of the fragmentation pathways in the mass spectrum of phenacetin, Garland had synthesized various ^{13}C labelled analogues of the compound. One of these was enriched to 60% in ^{13}C at the methylene carbon of the ethoxy group. This carbon of phenacetin is the one which should be hydroxylated by the microsomal enzymes to become the hemiacetal carbon, as shown in Figure 1. The ultimate fate of the ^{13}C -labelled carbon will be that of the carbonyl carbon of the

acetaldehyde released during the final step. Availability of this ^{13}C -labelled phenacetin suggested the possibility to observe the hemiacetal directly by ^{13}C NMR.

A number of other considerations supported the use of NMR. Firstly, the experimental conditions cannot be considered as harsh, in that the observation of the NMR signal takes place while the components are reacting in vitro in a glass tube. The magnetic field and three radio frequency bands can all be assumed to have no effect on the course of the metabolism. Secondly, the chemical shift range of ^{13}C is large and the shifts are very sensitive to the nature and degree of substitution; a large difference in chemical shift could be expected for the ^{13}C label in the phenacetin molecule and in the hemiacetal. This large chemical shift range, along with the one carbon-one resonance condition of proton-decoupled ^{13}C spectra, would help disperse the resonances of the sample over a range wider than that of ^1H NMR, and would allow a relatively easy assignment of the signals. This will be important since the final reaction mixture would be composed of many components: phenacetin, the microsomal material, (of unknown composition), the NADPH energy generating system (composed of glucose-6-phosphate, nicotinamide adenine dinucleotide phosphate and glucose-6-phosphate dehydrogenase), and ethylene diaminetetraacetic acid (EDTA).

In the ^{13}C spectrum the enzymic protein should give very broad signals of low intensity while the remainder of the carbons should present a spectrum which can be analyzed using standard chemical shift correlation charts.

The one factor which could prevent observation of the hemiacetal signal is the lifetime of its existence. Hemiacetals are rarely stable enough to be isolated, although when hydrated in aqueous solution they are stabilized. The ^{13}C enrichment greatly increases the chances of observation of the hemiacetal carbon since it takes many fewer transients to achieve the same signal-to-noise (S/N) ratio as in the natural abundance spectrum. The sensitivity of the labelled phenacetin is also essential because it will be necessary to work with small amounts of sample -- there is only limited volume in the receiver coil area of a 10 mm NMR tube, and the quantity of microsomal protein, substrate and cofactors that can be placed in this volume is finite. However, all these advantages of ^{13}C NMR and enrichment can be nullified if the hemiacetal is too rapidly converted to the final products.

Given all these factors, this portion of the thesis details the ^{13}C NMR experiments performed to observe the hemiacetal intermediate which is proposed to be formed during the in vitro metabolism of phenacetin by rat liver microsomes.

Chapter 2

MATERIALS AND METHODS

A. Chemicals

The 15.8 mg of ^{13}C -labelled phenacetin, p-[1- ^{13}C] ethoxyacetanilide, was the kind gift of Dr. W.A. Garland who synthesized it by refluxing potassium carbonate, p-hydroxyacetanilide and $\text{CH}_3^{13}\text{CH}_2\text{I}$ in acetone (26). The isotopic purity of the compound was determined by electron impact mass spectrometry and was found to be 60%.

The D-glucose-6-phosphate (monosodium salt), the nicotinamide adenine dinucleotide phosphate (monosodium salt; NADP), and the glucose-6-phosphate dehydrogenase (Type XII from Torula yeast) were purchased from the Sigma Chemical Company.

The ethylenediaminetetraacetic acid (anhydrous; EDTA), magnesium chloride (hexahydrate) and the various buffer components were common biochemistry laboratory shelf chemicals.

B. Solutions

The stock solution of the ^{13}C -enriched phenacetin was prepared by weighing out 5.00 mg of the material into a vial and adding 0.20 ml ethylene glycol- d_4 ($\text{HO}-\text{CD}_2-\text{CD}_2-\text{OH}$)

and 1.30 ml D₂O. The ethylene glycol was added to help solubilize the compound, and the deuterated form of the solvent was used to suppress its ¹³C resonance from appearing in the spectra of the in vitro reaction mixtures. This stock solution was kept refrigerated, and prior to use in an experiment it was necessary to sonicate and heat the solution for about 2 hours in order to solubilize the phenacetin. The concentration of this stock solution is 3.33 mg/ml or $(1.85) \times (10^{-2})$ M.

The 250 units of crystallized and lyophilized glucose-6-phosphate dehydrogenase (where one unit is defined at that amount which will oxidize 1.0 μ mole of glucose-6-phosphate to 6-phosphogluconate per minute at pH 7.4 at 25°C in the presence of NADP) was reconstituted by the addition of 0.250 ml D₂O. The concentration of this solution is 1000 units/ml. The solution was stored at -18°C, and when needed it was allowed to thaw at room temperature prior to use.

A 1.15% KCl solution in 0.05 M Tris HCl buffer, pH 7.4, was made by standard methods. The buffer used for the NMR in vitro reaction mixtures was a 0.05 M phosphate buffer, pH 7.4, which had been prepared using D₂O instead of H₂O.

C. Rat Liver Microsome Preparation

Two separate preparations of the rat liver microsomes were carried out, both of which followed the procedure now detailed.

Two Sprague Dawley rats (179 and 168 g in the first preparation, and 160 and 158 g in the second preparation) were given an intraperitoneal injection of 3-methylcholanthrene suspended in corn oil (16.0 mg in 2.0 ml). Each animal was given an amount (typically ca. 5 mg) proportional to receiving 25 mg of 3-methylcholanthrene per 1 kg body weight. This was carried out for the purpose of inducing the microsomal enzymes and increasing the phenacetin metabolism (27).

Twenty-four hours later the two rats were sacrificed by stunning and decapitation. After rapid excision and removal of all connecting tissue, the livers were placed on ice and weighed. The weight of livers in the first preparation was 16.6 g and in the second 16.7 g. The liver tissue was washed in 0.9% NaCl (pre-cooled to 4°C) in order to remove any remaining blood, fur, etc. They were next placed in three volumes (3 x liver weight, ca. 50 ml) of 1.15% KCl in 0.05 M Tris HCl buffer, pH 7.4 and 2°C, and finely minced with scissors. This suspension was then homogenized in a Teflon Potter-Elvehjem homogenizer which had been cooled to 2°C. The original

suspension was divided into ten portions for this step, and each one required 10-15 strokes in order to achieve the necessary homogenization. During the entire process the homogenizer was kept surrounded by ice water at 1°C to prevent any heating of the suspension and subsequent denaturation of the enzymes.

The homogenate was then centrifuged at 3°C at 9,200 x g for twenty minutes using a Sorval RC2-B centrifuge and SS-34 rotor (8750 rpm). Upon completion the cellular debris, whole cells and blood were a thick sludge at the bottom of the tubes, while the supernatant was a red suspension of moderate viscosity.

This supernatant was then divided into two equal portions and placed in ultracentrifuge tubes (tops assembled and tightened to 100 ft-lbs torque). The remaining volume in each tube was filled with cold (2°C) 1.15% KCl in 0.05 M Tris HCl buffer, pH 7.4. The tubes were placed in a pre-chilled TI-60 rotor and spun at 113,000 x g (40K rpm) at 2°C for 60 minutes. Upon completion the supernatant was a clear red and the pellet was a gelatinous translucent red material.

The two pellets were scraped and washed out with 1.15% KCl in 0.05 M Tris HCl buffer, pH 7.4, 2°C (about 30 ml total volume). The material was resuspended by homogenizing in a cold Teflon Potter-Elvehjem apparatus (six strokes and homogenizer surrounded by ice).

The resulting suspension was again centrifuged at 113,000 x g (40K rpm) for 60 minutes at 2°C using the same apparatus and procedure as previously employed. The supernatant, now a clear yellow, was discarded; the pellets were a gelatinous translucent red. To remove any remaining Tris (so that it would not appear in the ^{13}C NMR spectra) each pellet was washed with 1.15% KCl at 2°C and the wash solution discarded.

The microsomal pellets were removed by scraping and with a washing of 10-15 ml of 0.05 M phosphate buffer in D_2O , pH 7.4, at 2°C. These were resuspended via the chilled Teflon Potter-Elvehjem homogenizer.

Protein was determined in the resulting solution by the Lowry assay (28) using bovine serum albumin as a standard.

The microsomal protein concentration was found to be 29.3 mg/ml and 32.9 mg/ml, respectively, in the first and second preparations of the microsomes. Using the 0.05 M phosphate buffer in D_2O , pH 7.4, these were diluted to a final protein concentration of 10.0 mg/ml. The microsomal solutions were stored as 1 ml aliquots in individual vials, each one being fast frozen in liquid nitrogen and stored at -60°C.

When performing an experiment, the vials containing the required amount of microsomal protein (usually 15 to 50 mg) were removed and allowed to thaw at room temperature. It was again necessary to spin down these

solutions so that the microsomes could be added to the reaction mixture as a "solid". This concentrating was necessary because these solutions were 10 mg/ml, and the final volume of the in vitro experiment in the NMR tube could only be 1.0-1.5 ml in order to have sufficient ^{13}C sensitivity with the amounts of components to be used. The contents of the vials were placed in one screw-top small-volume centrifuge tube, diluted with cold 0.05 M phosphate buffer in D_2O , pH 7.4, until the tube was full, and centrifuged at $100,000 \times g$ (40K rpm) for 60 minutes at 2°C immediately prior to use. The supernatant was discarded and the pellet added directly to the NMR tube. A very small amount of the gelatinous pellet (1-5% by weight) would be lost in the transfer.

D. In Vitro Reaction Mixtures

As a guide to the quantities of the various components that would have to be used in the in vitro reaction mixture, the relative portions used by Garland in his studies (21) were employed. One such reaction mixture based upon the kinetic experiment with 0.10 M phenacetin is shown in Table 2. However these reaction mixtures were designed for detection by mass spectrometry, a technique with sensitivity orders-of-magnitude better than natural abundance ^{13}C NMR. To compensate for this,

Table 2

The composition of the in vitro reaction mixture for the microsomal metabolism of phenacetin

Amounts Used By
Garland et al. (2)
Typical Amounts Used
in ^{13}C NMR Experiments

Components	(Final Volume = 1.5ml)		(Final Volume = 1.5ml)	
	mg	mole	mM	mg
NADP	0.765	1	0.667	7.65
glucose-6-phosphate	2.821	10	6.667	28.21
glucose-6-phosphate dehydrogenase	1 unit	-	-	10 units
EDTA	0.4384	1.5	1.0	4.38
MgCl ₂	0.9149	4.5	3.0	9.15
microsomal protein	1.5	-	-	15-50
phenacetin	0.027	0.15	0.10	0.566

mM

mole

mg

mM

mole

mg

mM

mole

mg

the reaction must be scaled up by ten times while holding the final volume to 1.5 ml.

The one important signal which must readily be observable is that of the phenacetin. While in the 10 x scale-up the presence of phenacetin at 1.0 mM would not be sufficient to observe its natural abundance ^{13}C spectrum, the 60% ^{13}C -enriched carbon will give a strong resonance. Thus for equal amounts of phenacetin and its enriched analogue, the ^{13}C -enriched methylene carbon will have an intensity 54.5 ($60 \div 1.1$) times that of the natural abundance one. The 0.27 mg of ^{13}C -enriched phenacetin will give a signal for the methylene carbon which would be equivalent to that obtainable at natural abundance with 14.7 mg of phenacetin. This signal is readily observable in the ^{13}C NMR experiment.

The actual amounts of the various components used in a typical in vitro experiment are shown in Table 2. Due to the unknown lifetime of the hemiacetal to be observed, the amount of enriched phenacetin used was approximately doubled from that previously discussed. In this way the time necessary to observe the NMR signal from phenacetin, the hemiacetal or the final product was reduced a further 75%; the chances of observing the desired intermediate were increased. To ensure the complete metabolism of this amount of phenacetin the

quantity of microsomal protein also had to be increased (different degrees in different experiments) over the amount suggested by the scale up of Garland's experiments.

The NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ and EDTA were weighed directly into the NMR tube. The gelatinous pellet of microsomal protein was transferred into the tube with a spatula. After addition of 1.0 ml of the 0.05 M phosphate buffer in D_2O , pH 7.4, and 0.17 ml of the 3.33 mg/ml stock solution of phenacetin, the final volume was near 1.5 ml. The uncertainty is ± 0.1 ml and arises due to the unknown volume occupied by the microsomes.

The reaction mixtures were vortexed to homogeneity at all stages. After addition of the buffer, O_2 was blown onto the solution for $\frac{1}{2}$ minute (the microsomal enzymes utilize O_2 as an oxygen source, vide infra.) When the reaction mixture for the experiment was complete a Teflon anti-vortex plug was inserted and the NMR tube sealed with a cork stopper and Parafilm.

As the experiments progressed, minor modifications were made to this procedure; the order of addition of the reaction components and the amounts of some reagents were altered. These will be noted in the Results section as they are encountered.

E. Spectrometer Operation

All ^{13}C NMR spectra were obtained at 20 MHz, on a Varian CFT-20 pulse Fourier transform spectrometer which utilized a Varian 620/L data system. The spectra were recorded with broad-band noise-modulated proton decoupling (centered at 5 ppm in the ^1H spectrum) and at a temperature of 33°C . The transmitter was positioned near $\delta=0$ (relative to internal tetramethylsilane, TMS), and a sweep width of 4505 Hz was used to cover the entire ^{13}C spectrum of 225 ppm. The data acquisition time was 0.400 s (3604 data points); the pulse flip angle was 43° (90° pulse width required a 25 μs pulse). Prior to sampling and storage of each transient, the signal was passed through a single side band crystal filter with a bandwidth of 8000 Hz. After sufficient accumulations each FID was zero-filled to give a 4096 data point FID (no sensitivity enhancement weighting was applied), apodized and subsequently Fourier transformed. The heteronuclear field frequency lock was obtained from the deuterium of the D_2O solvent.

Off-resonance decoupling experiments were performed using the same data acquisition parameters.

The ^1H decoupler was left at full power but the noise modulation was removed. The decoupler was centered 3-5 ppm to high field of TMS.

All ^{13}C chemical shifts, δ , are reported as ppm downfield from TMS and are obtained directly from a computer subroutine.

Chapter 3

^{13}C NMR INVESTIGATION FOR THE HEMIACETAL INTERMEDIATE OF THE MICROSOMAL METABOLISM OF PHENACETIN

A. Introduction

In this chapter the experimental results from the ^{13}C NMR investigation of the in vitro metabolism of phenacetin by rat liver microsomes are presented. The reader will first be acquainted with the various reaction components and their functions, and then the results will be detailed.

Section B will explain the workings of the rat liver microsome in terms of the cycle by which its cytochrome P-450 oxygenating enzymes achieve hydroxylation. NADPH, a necessary cofactor for their functioning, is generated in the in vitro mixture by a three component system, the subject of Section C. Included in these two sections will be the spectra which would arise from each system. In this way a composite ^{13}C spectrum of the in vitro reaction mixture prior to addition of phenacetin and its metabolism will be constructed.

Section D will consist of the spectra of the ^{13}C -enriched phenacetin and of the in vitro mixture immediately after its addition. Finally, spectra of the metabolism endproduct, acetaldehyde, will be considered.

All of the spectra of this section will then be investigated for evidence for the microsomal metabolism of phenacetin as actually having proceeded as expected.

In section E attention will be turned towards the object of the experiments, the hemiacetal intermediate. Theoretical calculations will be made to predict the chemical shift value of the labelled carbon.

The final sections, F, G, H and I, present the ^{13}C NMR results from four separate in vitro experiments. Included in each one are the procedural changes (and their consequences) made while the experiments were in progress. The conclusions regarding the existence of the hemiacetal as an intermediate in the metabolism of phenacetin are presented.

B. Microsomal Enzyme System

The word "microsomes" originated in the field of microscopy and referred to the fact that these cellular components could not be visualized with light microscopy. They were "submicroscopic particles" or "microsomes".

The term is a working definition and does not refer to a particular cellular particle. Rather, it describes a cellular fraction obtained after a defined sequence of high-speed centrifugations of disrupted

cells from which large cellular components (nuclei, mitochondria and cellular debris) have been removed. The details of such a procedure can be found in Section C of Chapter 2, Material and Methods.

Microsomes are composed of endoplasmic reticulum with and without the RNA storage granules, the ribosomes. As was done in these experiments, the organ most often chosen from which to isolate the microsomes is the liver. While other tissue can be subjected to the same isolation procedure, use of liver leads to a highly homogeneous preparation compared to that from other organs.

The enzyme system of the microsomes have not been precisely characterized -- the number of different enzymes is large. Relevant to the metabolism being studied here is one group of mixed-function oxidases found in the endoplasmic reticulum. They constitute a membrane-bound nonphosphorylating electron-transport system capable of hydroxylation and desaturation reactions. In the liver an important electron-transport system is the one referred to as P-450. This consists of the flavo-protein NADPH-cytochrome P-450 reductase and the specialized cytochrome, cytochrome P-450.

The mechanism which has been postulated for the hydroxylation of organic substances by the liver microsomal P-450 system is shown in Figure 3 and now

Figure 3. Proposed mechanism for the hydroxylation of organic substances by the P-450 enzyme system.

detailed (29). The first step involves the substrate, A, combining with the Fe^{+3} form of the flavoprotein. This is converted to the Fe^{+2} form by one electron of NADPH being transferred to it via the flavin adenine dinucleotide (FAD). This completely reduced form of the flavoprotein reacts with molecular oxygen, O_2 , which subsequently picks up a second electron from NADPH via FAD. In this process the bound oxygen is converted to the O_2^- radical. An internal oxidoreduction occurs with the O_2 oxygen atoms ending up in H_2O and in the hydroxylated substrate, A-OH. This also regenerates the free Fe^{+3} form of the P-450. In some cases an iron-sulphur protein, adrenodoxin, transfers electrons from the FADH_2 to the P-450.

This electron transport enzyme system of liver microsomes catalyzes the hydroxylation of a wide variety of biologically important molecules. These include steroids, fatty acids, squalene and certain amino acids. As well, various pharmaceutical drugs, such as phenobarbital, morphine, codeine, phenacetin, amphetamines and carcinogenic polynuclear aromatic hydrocarbons are handled by the same microsomes. The hydroxylation of these drugs is generally considered to be part of the body's metabolism of these normally foreign materials.

3

One of the ways to increase the activity of this enzyme system is through induction with one of the above drugs it would normally hydroxylate. Administration of a barbituate such as phenobarbital leads to a great increase in the concentration of the flavoprotein and P-450 in the liver of the animal. It has been observed that the smooth portions of the liver endoplasmic reticulum undergo rapid growth (this is thought to be a defensive or protective adaptation). For the microsomes used in these experiments, induction was achieved with 3-methylcholanthrene injected (in corn oil) into the abdominal cavity 24 hours prior to sacrifice. This compound was chosen since it was found that induction of this same enzyme system for metabolism of the carcinogen benzo[a]pyrene was ten times greater with 3-methylcholanthrene than with phenobarbital (30).

Due to the high molecular weights of the microsome components, the great diversity of compounds, and the low concentration of any one component, the ^{13}C spectrum of such should be very weak. The relatively slow motions of these large molecules in solution lead to a partial breakdown of the "extreme narrowing limit" approximation. The increased T_1 's and decreased NOE's and T_2 's manifest themselves in low intensity, broad resonances. A typical spectrum from the prepared rat

liver microsomes is shown in Figure 4, for which 100,000 transients were taken of 65 mg of protein in 1.0 ml of 0.05 M phosphate buffer in D_2O , pH 7.4. There are four broad resonances visible: one centered near $\delta = 129.1$ (aromatic carbons); another centered at $\delta = 55.2$ (substituted aliphatic carbons); the very broad continuum from $\delta = 40-22$ (the aliphatic carbons); and the last centered about $\delta = 15.2$ (the aliphatic methyl carbon resonances).

As will be seen in the spectra of the in vitro reaction mixtures, these protein resonances will be of weak intensity compared to the sharp intense lines of the low molecular weight organic compounds. Also, these weak background resonances of the enzyme system fortunately occur in regions in which they do not interfere with or obscure any important resonances.

C. NADPH Generating System

As seen in the preceeding section, the microsomal enzyme requires NADPH as an electron donor in order to carry out the substrate hydroxylation. Rather than add NADPH directly into the reaction mixture, it is customary in such cases to generate in vitro using an enzyme catalyzed biochemical reaction. Consideration of the reactants in the microsomal P-450 enzyme system

Figure 4. ^{13}C NMR spectrum of rat liver microsome preparation.

and in the NADPH generating system, led to an obvious, inexpensive system capable of generating unlimited quantities of NADPH.

The way to accomplish this is to generate NADPH from NADP, the reaction shown in Figure 5. This is the reverse of the reaction occurring in the microsomes where NADPH is converted to NADP to supply the electrons for the hydroxylation. What one wishes to set up is a cycle. When the phenacetin is hydroxylated, the cofactor NADP will be produced and this will immediately be involved with the second enzyme system (to be described) to yield more NADPH as a cofactor product. This cycle will stay operational as long as there are substrates (i.e. phenacetin and glucose-6-phosphate, vide infra) for the main enzyme reactions.

The reaction commonly chosen for the generation of NADPH is the conversion of D-glucose-6-phosphate to D-glucose- δ -lactone-6-phosphate which in turn is hydrolyzed to 6-phospho-D-gluconate. This sequence is shown in Figure 6. The dehydrogenation of carbon 1 of the pyranose form of the glucose-6-phosphate is the first step and this is catalyzed by glucose-6-phosphate dehydrogenase. During this reaction NADP is converted to NADPH. This first step is readily reversible.

Figure 5. The conversion of NADP to NADPH.

Figure 6. Enzymatic degradation of glucose-6-phosphate to 6-phospho-D-gluconate.

The second step, the hydrolysis of the lactone to the 6-phosphoester, is rapid and spontaneous ($\Delta G^{\circ} = -5$ kcal/mole). In some plants and many species of bacteria, the enzyme gluconolactonase serves to catalyze this reaction, using Mg^{+2} as a cofactor. Overall the equilibrium of the two reactions lies to the right and thus favors the formation of NADPH.

The choice of this reaction for the conversion of NADP to NADPH is advantageous for a number of reasons. First, as already mentioned, it has an equilibrium in favor of the products. Second, since NADP-NADPH are involved in a cycle, the real "energy source" or chemical being expended during the reaction will be glucose-6-phosphate, an inexpensive biochemical. Third, the reaction is well understood due to thorough characterization. Fourth, the enzyme glucose-6-phosphate dehydrogenase is inexpensive and can be obtained in high purity and specificity.

In all the in vitro reactions, a large excess of glucose-6-phosphate has been used. The 100 μ moles added represents a tenfold excess over the 10 units of the dehydrogenase enzyme used. For maximum activity of the enzyme a minimum twofold excess, or 20 μ moles, of glucose-6-phosphate is needed. The excess used also insures that an adequate supply of NADPH can be produced

from any NADP present. A high concentration of Mg^{+2} has been used since the manufacturer found it increased the reaction rate. The EDTA is added to complex any heavy metals which would inhibit the action of any of the enzymes.

The ^{13}C spectrum of this NADPH generating system will be dominated by the resonances of the glucose-6-phosphate. As indicated in Table 2, its final concentration will be near 0.07 M and thus easily seen in the ^{13}C NMR spectra, most of which were accumulations of 100K transients. The glucose-6-phosphate dehydrogenase is present in negligible amounts (10 units represents 24 μg), and the resonances of NADP or NADPH, depending on the two controlling reactions, never appeared at the positions reported in the literature (31).

The spectrum of the glucose-6-phosphate in the 0.05 M phosphate buffer in D_2O , pH 7.4, is shown in Figure 7. The two resonances at low field, $\delta = 97.10$ and $\delta = 93.30$, are those of C-1 of the β and α forms, respectively. The highest field resonance belongs to C-6, and the fact that it appears split (or broadened, depending on number of data points) is an indication of long range ^{31}P coupling. The remainder of resonances between 70.3-77.1 ppm belong to carbons C-2 to C-5 of the α and β forms.

Figure 7. ^{13}C spectrum of glucose-6-phosphate in 0.05 M phosphate buffer, pH 7.4, in D_2O .

Figure 8 illustrates the spectrum of the completely assembled NADPH generating system: glucose-6-phosphate, NADP, glucose-6-phosphate dehydrogenase, EDTA and MgCl_2 (according to the quantities in Table 2) in 1.5 ml of 0.05 M phosphate buffer in D_2O , pH 7.4. As predicted, almost all the visible resonances belong to glucose-6-phosphate. There has been some minor shifting of resonances but generally these are less than 1 ppm. The largest one is a 3.2 ppm upfield shift for the broad C-6 resonance. Physical interactions with the various metal ions or cofactors is thought to lead to this.

There is one weak resonance at ca. $\delta = 101$ which is just visible in Figures 7 and 8. This broad peak appears more intense in some spectra and since it occurs near the region of the hemiacetal (vide infra), it is necessary to point out that it is introduced with the glucose-6-phosphate. It is most likely due to the C-1 of a minor impurity of glucose-1-phosphate in the commercial glucose-6-phosphate.

At $\delta = 60.5$ and $\delta = 56.5$ of Figure 8, there are narrow resonances of low intensity which are not present in all spectra of the in vitro mixtures. These are most likely resonances of EDTA; the $\delta = 60.5$ belongs to the methylene carbon α to the carbonyl, and $\delta = 56.5$ the peak of the central methylene carbons.

Figure 8. ^{13}C NMR spectrum of complete NADPH generating system.

The two should display a 2 to 1 intensity ratio, which they are observed to do. The carbonyl resonance is not seen because of the pulse conditions which suppress such slowly relaxing carbons. The fact that EDTA is involved in complex equilibria depending on metal content and pH might explain the fact that the peaks do not always appear.

Figure 8 is a good indication of what the spectra of the in vitro reaction mixture will look like. The spectrum of the microsomal protein, Figure 4, must be superimposed, and the phenacetin and final product resonances added.

D. Deethylation of ^{13}C -Labelled Phenacetin In Vitro

The first item to ascertain for the assembled in vitro system is that the deethylation of phenacetin is actually occurring. The proof of this will be the disappearance of the methylene resonance of ^{13}C -labelled phenacetin and the appearance of the ^{13}C -labelled carbonyl of acetaldehyde, the final product.

The spectrum of 0.225 M unlabelled phenacetin in CDCl_3 is shown in Figure 9. CDCl_3 was chosen as a solvent since there is no problem with solubility (as there is in D_2O). The following indicates the assignment of the resonances from the CDCl_3 solution.

Figure 9. ^{13}C NMR spectrum of phenacetin in CDCl_3 .

These have been made using additivity relationships and the model compounds ethoxybenzene and acetanilide.

The chemical shifts of a dilute aqueous solution of phenacetin will be slightly different, as will be noted for the methylene carbon resonance (vide infra). This is explainable in terms of different solvent interactions and the reporting of chemical shifts to internal and external TMS for CDCl_3 and D_2O respectively.

The aqueous stock solution of ^{13}C -labelled phenacetin (preparation detailed in B, Chapter 2) has the spectrum shown in Figure 10. The chemical shift of the methylene carbon is $\delta = 65.79$, a 2.15 ppm downfield shift. The spectrum clearly demonstrates the fact that only the methylene carbon of phenacetin has been labelled. None of the other resonances are visible despite the fact that in Figure 9 the resonances of the protonated aromatic carbons are approximately twice the intensity of that of the methylene carbon.

Figure 10. ^{13}C NMR spectrum of the stock solution of ^{13}C -labelled phenacetin.

The final product, acetaldehyde, has the ^{13}C spectrum shown in Figure 11. The Figure 11.A is for a high concentration of acetaldehyde in D_2O , while a very dilute solution in the 0.05 M phosphate buffer in D_2O , pH 7.4, gives the spectrum shown in Figure 11.B. Neither of the concentrations can be accurately determined since the boiling point of acetaldehyde is 20.8°C . There is considerable rapid evaporation even when transferring chilled acetaldehyde (2°C) with pre-chilled pipets. Once it is added to the aqueous medium there is a further evaporation as evidenced by the strong odor coming off the solution. The terms "high concentration" and "very dilute" are arrived at by consideration of the number of transients needed to acquire each spectrum: Figure 11.A has a $\text{S/N} = 64$ from 3900 transients, and Figure 11.B a $\text{S/N} = 11$ from 25,000 transients (as obtained using the $\delta = 24.4$ resonance).

Instead of observing two resonances in these aqueous solutions of acetaldehyde, four are present. The reason for this is that acetaldehyde is extensively hydrolyzed in water to a hydrated gem-diol form. The equilibrium that is established and the chemical shifts of the two forms are shown in Figure 12. In Figure 11 it is evident that the two resonances for the diol form are more intense than those of the free acetaldehyde.

Figure 11. ^{13}C NMR spectra of acetaldehyde in aqueous solution.

A. Concentrated in D_2O

B. Dilute in 0.05 M phosphate buffer in D_2O , pH 7.4

δ_{C}

Figure 12. The equilibrium of acetaldehyde in aqueous solution.

ACETALDEHYDE

$\delta=31.27$

$\delta=208.12$

gem-DIOL

$\delta=24.33$

$\delta=89.32$

Since the relaxation times of the methyl carbons and those of the carbonyl and diol carbon should be relatively similar, the intensities indicate that the equilibrium has been established in favor of the diol form.

When the acetaldehyde is produced as the end product of the metabolism of ^{13}C -labelled phenacetin, its resonance intensities will be altered since the final fate of phenacetin's ^{13}C -labelled carbon is the carbonyl of acetaldehyde. Due to the hydration and equilibrium, the majority of this will end up on the methine carbon of the diol form. Thus, the resonances at $\delta = 208.12$ and $\delta = 89.32$ should be greatly intensified compared to those of the methyl carbons in the two forms of acetaldehyde.

The purpose of the first in vitro experiment was to observe the disappearance of phenacetin's ^{13}C -labelled methylene resonance (Figure 10) and the subsequent appearance of the two forms of ^{13}C -labelled acetaldehyde (the $\delta = 208.12$ and $\delta = 89.32$ of Figure 11). The in vitro reaction mixture was assembled as detailed in Section D of Chapter 2, except for the following two modifications: (i) the glucose-6-phosphate dehydrogenase and the microsomal protein were not added until a ^{13}C spectrum of the reaction mixture without

proteins and enzymes present was obtained; and (ii) as originally suggested by the tenfold scaleup of Garland's experimental quantities (Table 2), the original addition of microsomal protein was 15 mg.

Figure 13 is the spectrum of the mixture prior to addition of any proteins or enzymes. As expected, only the resonance of ^{13}C -labelled phenacetin, $\delta = 65.79$, and those of glucose-6-phosphate are observed -- superimposing Figures 7 and 9 would give much the same spectrum. The important point to note in Figure 13 is the relative intensities of the peaks. Since metabolism is not taking place in this mixture devoid of the enzymes, and since it contains the same relative amounts of phenacetin and glucose-6-phosphate as will be used in the functioning metabolic system to be studied, the relative intensities of the two compounds represent the relative intensities of zero-time of the metabolic deethylation reaction. The ^{13}C -labelled phenacetin is slightly more intense than any resonance of the glucose-6-phosphate. When the reaction is allowed to proceed this should be reversed; the phenacetin will be metabolized and its resonance will disappear relative to those of glucose-6-phosphate. The fact that it takes two glucose-6-phosphate for a deethylation of one phenacetin is overcome by the large excess of the

Figure 13. ^{13}C NMR spectrum of in vitro reaction mixture #1 prior to addition of microsomes.

glucose-6-phosphate added (67 times the number of moles of phenacetin).

After addition of the glucose-6-phosphate dehydrogenase and the 15 mg of microsomal protein, three spectra were taken over the next 26 hours. These are shown in Figure 14; Figure 14.A is the spectrum obtained from 0 to 8.5 hours, Figure 14.B that from 18.0 to 20.5 hours, and Figure 14.C that from 21.0 to 26.0 hours. A minor feature to note in these spectra is that upon assembly of the complete metabolic reaction mixture, the C-6 resonance of glucose-6-phosphate undergoes the 3.2 ppm upfield shift previously noted in Figures 7 and 8. This relocation to $\delta = 61.9$ is convenient in that the resonance from ^{13}C -labelled phenacetin, $\delta = 65.79$, is no longer obscured by a directly adjacent resonance at $\delta = 65.1$.

Relative to glucose-6-phosphates resonances, the intensity of the ^{13}C -labelled phenacetin resonance at $\delta = 65.79$ in Figure 14.A is approximately 50% less than that of the zero-time intensity in Figure 13. Whereas it was once larger than any glucose-6-phosphate resonance, it is now substantially smaller. Thus the deethylation metabolism of phenacetin appears to be operative. When one observes the $\delta = 65.79$ resonance in Figure 14.A, 14.B and 14.C, there is a very small

Figure 14. ^{13}C NMR spectra of in vitro reaction mixture #1 at various times after addition of 15 mg microsomal protein.

decrease in its intensity with time. It appears that the majority of the observed metabolism occurs very rapidly initially and then only very slowly after that.

To determine if there had been sufficient microsomal protein to carry out the rapid metabolism of all the phenacetin, two separate additions of 25 mg of microsomal protein were made to the in vitro mixture. The ^{13}C spectra for the reaction mixture with a total of 40 mg and finally 65 mg of microsomal protein are shown in Figure 15.A and 15.B, respectively. The four broad protein resonances observed in Figure 4 are now quite evident in the in vitro reaction mixture. The effect of this additional microsomal protein is the rapid and complete elimination of the ^{13}C -labelled phenacetin peak.* This is good evidence that the deethylating metabolism is operative.

The other proof for active metabolism, the appearance of the ^{13}C -labelled acetaldehyde resonances at $\delta = 208.12$ and/or $\delta = 89.32$, is missing from the spectra in Figures 14 and 15. However, upon unsealing the sample tube the odor of acetaldehyde was briefly

* The results of this experiment led to the modification of the original plans to only use 15 mg of microsomal protein. Instead, 50 mg was used to insure the total metabolism of the phenacetin used in all the metabolic experiments.

Figure 15. ^{13}C NMR spectrum of in vitro reaction mixture #1 with varying amounts of microsomal protein.

A. 40 mg microsomal protein

B. 65 mg microsomal protein

quite distinct. Thus, it was likely that the acetaldehyde produced in the deethylation metabolism was being vaporized into the void air space above the vortex plug in the NMR tube. Considering the boiling point of acetaldehyde, 20.8°C, and the probe temperature, 33°C, this explanation is reasonable. As the acetaldehyde in the reaction solution is vaporized, there is a disruption of the equilibrium which exists in aqueous solution between acetaldehyde and its hydrated gem-diol form, Figure 12. This equilibrium is shifted significantly towards the acetaldehyde form to try and maintain the equilibrium. Since the dead volume in the NMR tube is large and the amount of acetaldehyde produced is small (maximum of 3.14 μ mole with 100% metabolism of the phenacetin), all the acetaldehyde produced eventually ends up in the air above the vortex plug. The consequence is that the ^{13}C -labelled acetaldehyde signals are absent from the spectra.

To establish more firmly the production of acetaldehyde in the in vitro reaction mixture, the metabolic study was repeated at threefold higher concentrations. For this experiment, ^{13}C spectra were obtained prior to addition of any enzyme material and then after an addition of the glucose-6-phosphate dehydrogenase and 150 mg of microsomal protein. The two spectra were

near identical to those in Figure 13 and 15.B, respectively.

However, immediately after obtaining each spectrum, a thin layer chromatography (TLC) method was carried out to test for the presence of acetaldehyde above the vortex plug in the sealed NMR tube. Using a syringe inserted through the cork on the NMR tube, the air in dead space above the vortex plug was sampled, as in Figure 16.A. In the syringe was an aqueous solution of 2,4-dinitrophenylhydrazine, through which the air in the NMR tube was slowly bubbled as the syringe's plunger was pulled out. When the syringe was full it was tipped so that only the air in it was returned to the NMR tube. The withdrawal and return of the air from the dead space was repeated ca. 25 times.

Any acetaldehyde in the dead air space will react with the 2,4-dinitrophenylhydrazine while being drawn into and bubbled through the aqueous solution. The reaction which occurs is shown in Figure 16.B. The 2,4-dinitrophenylhydrazone derivative is commonly used to characterize aldehydes since it usually is a solid (32). For the air sample collected before the addition of the metabolizing enzymes, the aqueous solution remained perfectly clear. However, the air pulled into the 2,4-dinitrophenylhydrazine solution after the

Figure 16. Testing for acetaldehyde in the dead space of the NMR tube.

A. Sampling the air above the antivortex plug.

B. The 2,4-dinitrophenylhydrazone derivative of acetaldehyde.

addition of the enzymes and subsequent depletion of the ^{13}C -labelled phenacetin led to a slightly cloudy appearance. It is possible that this was caused by the precipitation of the acetaldehyde derivative.

For both samples, four equal portions of ethylacetate were used to extract the aqueous solution and to wash out the syringe. Both ethylacetate solutions now containing unreacted 2,4-dinitrophenylhydrazine and any hydrazone derivative, were concentrated down with nitrogen. Each sample was then spotted onto a TLC plate along with standards of 2,4-dinitrophenylhydrazine and the 2,4-dinitrophenylhydrazone derivative of acetaldehyde. The plate was run in the solvent system carbon tetrachloride:hexane:ethylacetate (10:2:1), sprayed with a 1:1 solution of 1M NaOH and ethanol, and then developed by heating.

Examination of this TLC plate* indicated that the standard of 2,4-dinitrophenylhydrazine and of the 2,4-dinitrophenylhydrazone derivative of acetaldehyde had R_f values of 0.47 and 0.34, respectively.

* The Polaroid photograph of this TLC plate was not treated and thus the image disappeared upon standing. The R_f values were obtained from the original plate prior to discarding.

The sample originating from the dead air space prior to addition of the enzymes showed only one spot with an R_f of 0.45. The air sample taken after the metabolic enzymes were added and the phenacetin resonance had disappeared from the ^{13}C spectrum showed two distinct spots. One was very pronounced and had an R_f value of 0.47, while the other was much less intense and had an R_f of 0.33.

Thus, there is evidence for acetaldehyde production when the complete deethylating metabolic system is assembled. Unfortunately ^{13}C NMR cannot determine if the acetaldehyde found in the dead air space is actually ^{13}C labelled. Had a technique such as GC-MS verified this, there would have been indisputable proof for the metabolism of the phenacetin by the rat liver microsomes. However, the evidence which is available -- (i) the disappearance of the resonance of ^{13}C -labelled phenacetin and no appearance of any new signal in the spectrum (implying the ^{13}C has left the solution or has been distributed to many new molecular environments), and (ii) the identification of acetaldehyde in the dead air space after the complete metabolic system has been assembled and allowed to incubate -- strongly suggests that the deethylating metabolism of phenacetin is operative in the system.

With the system functioning as expected, the investigation for the hemiacetal intermediate was now feasible.

E. Theoretical ^{13}C Chemical Shift of the Hemiacetal Intermediate

To assist the search for the resonance of the hemiacetal, it was desirable to know its approximate ^{13}C chemical shift. Thus, a literature search for and a theoretical prediction of the chemical shift value of the hemiacetal carbon was undertaken.

The ^{13}C NMR literature contains very few reports on hemiacetals, and certainly no chemical shift value for the specific one of interest. In the book by Wehrli and Wirthlin (33), there are two ranges of values which apply to the closely related hemiacetals, hemiketals, ketals, acetals and gem-diols. These are shown below.

89-99 ppm

94-106 ppm

Thus, it is likely that the carbon of the hemiacetal intermediate of phenacetin metabolism will appear in the same spectral region as do $\delta = 93.30$ and $\delta = 97.10$,

two of the prominent resonances of the assembled metabolic reaction mixture (see for example, Figure 15). These peaks were assigned to the α and β forms, respectively, of C-1 of glucose-6-phosphate. This carbon is one of the few examples of a hemiacetal carbon which appears in the literature. Some further examples of hemiacetals or carbons with two directly-bonded oxygen atoms are shown in Figure 17. Another example of a closely related compound is the gem-diol carbon of hydrated acetaldehyde (Figure 12) for which the chemical shift is 89.32. The limited number of examples of hemiacetal chemical shifts is attributable to their instability. They are generally reversibly formed and are rarely stable enough to be isolated. As is evident in these cited examples, hemiacetals are most often observed in a ring form (e.g. glucose-6-phosphate) and/or a form stabilized by solvation (as for acetaldehyde where the gem-diol carbon can only be observed in aqueous solution).

To predict more accurately the chemical shift of the hemiacetal intermediate evolving from the deethylation metabolism of phenacetin, it was necessary to turn to model compounds and substituent effects. Fortunately, the chemical shift of a carbon can often be accurately determined from these bases. In the

Figure 17. Examples of the ^{13}C chemical shifts of hemiacetals, acetals, hemiketals or ketals.

$$\delta = 99.5 \quad (34)$$

$$\begin{aligned} \delta_1 &= 90.0 \\ \delta_2 &= 104.0 \end{aligned} \quad (34)$$

$$\delta = 104.6 \quad (35)$$

$$\delta_{\text{C}8} = 89.3 \quad \delta_{\text{C}10} = 102.0 \quad (36)$$

$$\delta_{\text{C}8,10} = 99.8 \quad (36)$$

case of the hemiacetal of interest, there is an excellent set of compounds available for such a prediction.

The calculation begins by consideration of phenacetin as resulting from replacement of the hydrogen of the hydroxyl group of ethanol with the $-\text{O}-\text{NH}-\text{CO}-\text{CH}_3$ group, as shown in Figure 18.A. Therefore, the effect of this group on the chemical shift of the carbon directly bonded to the oxygen onto which this group is substituted is merely the difference between the ^{13}C chemical shifts of the methylene carbons of phenacetin and ethanol. These are indicated by the * in Figure 18.A.

In a similar manner, phenacetin's hemiacetal intermediate can be envisioned as arising from the substitution of this same $-\text{O}-\text{NH}-\text{CO}-\text{CH}_3$ group onto the oxygen atom of one of the hydroxyl groups of the gem-diol form of acetaldehyde. Figure 18.B indicates this. In this case though, the effect of the substitution of the $-\text{O}-\text{NH}-\text{CO}-\text{CH}_3$ group is known from the above consideration of phenacetin and ethanol. Coupling this information with the known ^{13}C chemical shift of the methine carbon of the hydrated form of acetaldehyde, $\delta = 89.32$ from Figure 11.B, allows for the calculation of the hemiacetal chemical shift.

For the calculation of the effect of the

$-\text{O}-\text{NH}-\text{CO}-\text{CH}_3$ group from phenacetin and ethanol, the chemical shifts of interest were considered in two solvents, D_2O and CDCl_3 . The chemical shift of the methylene carbon of phenacetin in CDCl_3 and D_2O was taken from Figures 9 and 10, while those of ethanol's methylene carbon were experimentally determined to be $\delta = 58.39$ and $\delta = 58.61$, respectively.* The effect of substitution of the $-\text{O}-\text{NH}-\text{CO}-\text{CH}_3$ group in the two solvents is then calculated to be:

	<u>D_2O</u>	<u>CDCl_3</u>
δ of phenacetin's methylene carbon =	65.79	63.64
δ of ethanol's methylene carbon =	<u>58.61</u>	<u>58.39</u>
∴ the effect of		
$-\text{O}-\text{NH}-\text{CO}-\text{CH}_3$ substitution =	7.18	5.25

- * The hemiacetal for which the chemical shift is to be predicted will occur at very low concentration. Thus, the chemical shifts of ethanol were determined from dilute samples ($<0.05\text{M}$). The chemical shift for the phenacetin in D_2O is from the dilute stock solution ($<0.02\text{M}$) of ^{13}C -labelled phenacetin. Only the value for phenacetin in CDCl_3 is from a concentration solution (0.225M).

The gem-diol of acetaldehyde does not exist in CDCl_3 , and thus its chemical shift in D_2O , $\delta = 89.32$, must be used for the two calculations of the hemiacetal chemical shift. Therefore, combining this value with the downfield shift of the $-\text{O}-\text{NH}-\text{CO}-\text{CH}_3$ group calculated above, the ^{13}C chemical shift of the hemiacetal is predicted to be:

	<u>D_2O</u>	<u>CDCl_3</u>
δ of methine carbon of <u>gem</u> -diol form		
of acetaldehyde (in D_2O)	= 89.32	89.32
effect of $-\text{O}-\text{NH}-\text{CO}-\text{CH}_3$ substitution =	7.18	5.25
	<u> </u>	<u> </u>
$\therefore$ predicted δ of hemiacetal =	96.50	94.57

Since the compounds used in this prediction are all closely related to phenacetin, the hemiacetal and the final metabolic products, the actual chemical shift of the hemiacetal should be very near to the calculated one. It is expected that the calculated value for the hemiacetal in aqueous solution will most accurately predict that of the hemiacetal formed in the incubation mixture. However, the presence of the buffer components, the organic cofactors and the enzymes may lead to some small shift away from the value calculate in D_2O above. The predicted region in which the hemiacetal carbon resonance should occur in the ^{13}C spectra of the assembled metabolic system is between

the two resonances of the C-1 of glucose-6-phosphate, $\delta = 97.10$ and $\delta = 93.30$.

There are two other criteria which any resonance falling at the predicted region must meet in order to be identified with the hemiacetal. Firstly, the intensity of the resonance must not be greater than that of the phenacetin resonance prior to metabolism by the microsomal enzymes. The T_1 of phenacetin's methylene carbon and that of the hemiacetal's methine carbon will both be dominated by a dipole-dipole mechanism ~~with~~ their directly-bonded hydrogen(s). Based on the inverse relationship of T_1 and the number of directly-bonded hydrogens, the T_1 of the hemiacetal methine should not be greater than twice that of the phenacetin methylene. Under the pulse conditions employed to obtain most spectra (see Section E of Chapter 2), and if equal numbers of ^{13}C -labelled phenacetin and hemiacetal carbons were present, the methine carbon of the hemiacetal should have a less intense resonance than that of the phenacetin methylene. The maximum intensity of the ^{13}C -labelled methylene of phenacetin will be like that in Figure 13, a spectrum taken prior to metabolism. Here, the resonance is slightly more intense than any glucose-6-phosphate resonance. Since the amount of glucose-6-phosphate

remains relatively constant, any resonance in the in vitro reaction mixture to be identified as that of the hemiacetal must not be more intense than those of the glucose-6-phosphate. The second criterion for a hemiacetal resonance is that it must give a doublet in an off-resonance decoupling experiment.

Thus, there are five events one hopes to observe in the ^{13}C NMR experiments to prove that the deethylation metabolism of phenacetin by rat liver microsomes proceeds via a hemiacetal intermediate.

In summary, these are:

- (i) ^{13}C -labelled methylene of phenacetin, $\delta = 65.79$.
decreases in intensity and hopefully
disappears
- (ii) ^{13}C -labelled acetaldehyde resonances, $\delta = 208.12$
and $\delta = 89.32$, appear
- (iii) a sharp resonance appears between $\delta = 94.57$
and $\delta = 96.50$
- (iv) this sharp resonance is less intense than the
largest resonance of glucose-6-phosphate
(most often that of C-6 at $\delta = 61.9$)
- (v) the sharp resonance becomes a doublet in an
off-resonance decoupling experiment.

F. Results of In Vitro Experiment #3

Upon reexamination of the spectra from the first two in vitro experiments, there was no evidence of any new resonance between $\delta = 89-99$ and definitely not at the predicted $\delta = 96$. These experiments were discussed in Section D of this chapter, and the spectra from the experiment #1 were included as Figures 13, 14 and 15. The spectra of experiment #2 have not been included in this thesis but are identical to those of experiment #1. In Figure 15.B there are new resonances in a region $\delta = 101-103$. When observed, these are always broad and very weak, and as pointed out in Section C, associated with the glucose-6-phosphate. If the spectra from all the experiments, #1-#6, are examined, the resonances in this $\delta = 101-103$ region appear and disappear indiscriminantly, and for this reason will not be discussed further.

The in vitro experiment #3 was performed with some procedural changes in the hope that the hemiacetal might be observed. First, the order in which the components were added was altered. Using 50 mg of microsomal protein, the metabolic system was assembled without the ^{13}C -labelled phenacetin. The spectrum of the resultant sample is shown in Figure 19.

Figure 19. ^{13}C NMR spectrum of in vitro reaction mixture #3
prior to addition of phenacetin.

The second procedural modification in experiment #3 involved the accumulation of the spectra immediately after addition of the phenacetin. On the assumption that the hemiacetal would reach maximum concentration soon after the deethylation metabolism began, the ^{13}C NMR spectrum was obtained with the faster acquisition time of 0.1 s (usually 0.4 s) and a lesser number of transients. Under these conditions it was hoped that a hemiacetal resonance would be observed in a greater number of individual transients and thus in the final transformed spectrum.

Prior to the addition of the phenacetin the spinner housing and tube depth guide were placed on the NMR tube containing the metabolic mixture which gave the spectrum in Figure 19. The sample was placed in the probe a ^2H lock obtained and the field homogeneity optimized. After the sample tube was removed and the ^{13}C -labelled phenacetin added, the following steps were carried out in ca. 20 s: an anti-vortex plug was inserted, the tube was corked and covered with Parafilm and inserted back into the magnet, a lock was obtained, and the rapid accumulation of the spectrum was begun. After ca. 1.5 hours of accumulation the spectrum was stored on magnetic tape and a long accumulation using the normal 0.4 s acquisition time

was started.

The spectra from the short and the long accumulations are shown in Figure 20.A and 20.B, respectively. By comparing the small residual amounts of unmetabolized phenacetin ($\delta = 65.79$) in the two spectra, one observes that the amount remaining after a short period of time immediately after addition of the phenacetin is not very different from the amount remaining after 24 hours. Thus, the deethylation metabolism occurs very rapidly. It is not surprising then that no resonance attributable to the hemiacetal can be observed in the rapidly accumulated spectrum. Further proof for the speed of the reaction is that Figure 20.A does not contain resonances of ^{13}C -labelled acetaldehyde -- even it boils out of the aqueous solution so rapidly that no trace is observed.

Thus, if the hemiacetal intermediate were being produced in this reaction solution, its lifetime was too short to allow observation by FT ^{13}C NMR. No further work was carried out in experiment #3.

Figure 20. ^{13}C NMR spectra of in vitro reaction mixture #3 at various times.

A. 0 - 1.5 hr.

B. 1.5 - 24 hr.

G. Results of In Vitro Experiment #4

The failure to observe the hemiacetal in the first three experiments suggested that a means had to be developed to keep the hemiacetal present in solution for a longer period of time. The method arrived at resulted from careful consideration of what was happening in the NMR tube during the in vitro metabolism.

Figure 1 is an oversimplification of the deethylation mechanism operative in these NMR experiments. First, the acetaldehyde does not occur solely in its native form in the aqueous reaction solution, but rather, as was explained in Section D, it is in equilibrium with its hydrated gem-diol form. The second oversight of Figure 1 is the fact that it only deals with the reactions occurring in the aqueous environment. It neglects to consider the events in one other part of the closed system in which the metabolism occurs. This is the air space above the antivortex plug in the stoppered NMR tube, a volume 7-9 times the size of the aqueous volume. As was shown by the TLC method employed in experiments #2, the final product of the metabolism, acetaldehyde, ends up in this air space. To be complete then, another equilibrium, that between acetaldehyde in solution and in gaseous form, must be considered. Figure 21 presents a revised and a more complete picture of what is actually happening in the

Figure 21. The complete description of the equilibria involved in the in vitro metabolism of phenacetin.

in vitro metabolic reactions run in the stoppered NMR tube.

Due to the very low boiling point of acetaldehyde (20.8°C), the room temperature of 33°C that the NMR tube is subjected to, and the large air space compared to aqueous volume, the second equilibrium discussed above is greatly shifted in favor of the acetaldehyde leaving the solution and residing in the air space. The effect of this is to cause shifts in the other equilibria towards production of acetaldehyde in solution. The gem-diol form is dehydrated back towards acetaldehyde and thus it has never appeared in any spectra in the previous experiments. The most important consequence is that any hemiacetal formed, regardless of its stability, will be rapidly converted to acetamidophen and acetaldehyde as this equilibrium also responds to the rapid vaporization of acetaldehyde.

The method for lengthening the stay of any hemiacetal became apparent upon consideration of all the above equilibria. The addition of acetaldehyde to the air space and the aqueous in vitro mixture should reverse these equilibria by Le Chatelier's principle*.

* Qualitatively, Le Chatelier's principle states that when an equilibrium system is perturbed, the equilibrium will always be displaced in such a way so as to oppose the applied change.

The effects of such additions can be predicted to be as follows:

- (i) Excess chilled acetaldehyde rapidly added on top of the antivortex plug of a freshly assembled in vitro mixture should quickly evaporate and saturate the air space in the tube once it is stoppered. The result of this may be to prevent loss from the aqueous solution of any acetaldehyde originating from the phenacetin. This ^{13}C -labelled acetaldehyde will now appear in the spectrum at $\delta = 208.12$ and $\delta = 89.32$, the resonance positions of the methine carbons of the two forms of acetaldehyde present in aqueous solution (Figures 11 and 12). In addition, the equilibrium between the final products and the hemiacetal may be altered so as to increase the lifetime and the amount of the hemiacetal in solution and thus allow its observation. The success of this approach and such resultant spectral manifestations depends on two factors. Firstly, the air in the dead space must become saturated very rapidly compared to the production of acetaldehyde via the metabolism. If this is not possible the acetaldehyde, and thus the

^{13}C label through which the hemiacetal must be observed, will leave the solution. Secondly, the seal between the antivortex plug and the NMR tube must be good and no liquid acetaldehyde should enter the center hole of the plug. Depending on these factors, some of the acetaldehyde in the in vitro solution may be unlabelled. This would lead to the observation of the methyl resonances of the two forms of acetaldehyde at $\delta = 31.27$ and $\delta = 24.33$. While this may not hinder observation of the hemiacetal (and in fact, probably assist in it, vide infra), the presence of the four acetaldehyde resonances would detract from the proof for the deethylation metabolism being operable in this system. If only the methine resonances are present the fate of the label and the viability of the assembled phenacetin metabolism system are obvious (as explained in Section D).

- (ii) The second manner in which chilled liquid acetaldehyde can be added to alter the equilibria is with a direct, rapid addition to the in vitro solution. Much of this acetaldehyde would

"boil" off and end up in the air-space above the antivortex plug. Due to this saturation of the air space and the excess acetaldehyde in solution (by which the equilibrium between the aldehyde and the gem-diol will mainly be setup, Figure 12), the hemiacetal should be greatly favored in its equilibrium with the final products. Further, if there is an excess of acetaldehyde in solution prior to the start of metabolism, the ^{13}C label will stand less of a chance of transferring right through and onto the acetaldehyde the only species by which it can escape the in vitro mixture. Thus, with the majority of the label in solution and with the hemiacetal species favored, this should be the optimum situation for the desired observation. The only problem this approach presents was already mentioned in the previous point: adding acetaldehyde directly to the in vitro solution will obscure the observation of labelled acetaldehyde, the one unambiguous proof for the correct functioning of the metabolic system. Only if the intensities of the ^{13}C -labelled resonances at $\delta = 208.12$ and $\delta = 89.32$ are much greater than those of the

methylys will such a proof be possible with this addition of acetaldehyde into the solution.

- (iii) The third manner of altering the equilibria with addition of acetaldehyde is a variation of the preceding one. In this case, the chilled liquid acetaldehyde is added into the in vitro solution and also on top of the plug after it has been inserted. Once the tube is stoppered, the acetaldehyde on the plug saturates the air space and minimizes the loss of the acetaldehyde added to the solution. Thus, less acetaldehyde will have to be added to the solution, a desirable situation to prevent any damage to the metabolic system. The spectra which one expects from the in vitro system treated in this way are the same as those predicted for addition of acetaldehyde only to the aqueous solution (vide supra).

The first attempt at using any of these approaches to facilitate observation of the hemiacetal was in experiment #4. Here the chilled acetaldehyde was added directly to the aqueous reaction mixture. The problem that was encountered with the addition of

the acetaldehyde was that it would be very difficult if not impossible to quantitate the amount added. Even with cold acetaldehyde, a prechilled pipet and the precaution of not directly handling them or the NMR tube, acetaldehyde evaporation was very rapid. It was not possible to chill the NMR tube and antivortex plug since the Teflon plug would contract and sink into the solution. Thus, if a drop of acetaldehyde touched the NMR tube ca. > 2 cm above the aqueous level, it was observed to evaporate completely before any could enter the aqueous environment. Even when the chilled acetaldehyde was added directly into the in vitro mixture and the plug rapidly inserted and the tube stoppered, the acetaldehyde odor was very strong.

This difficulty was very evident during the preparation of the metabolic mixture in experiment #4. Into the NMR tube was first placed the NADPH-generating system, cofactors, buffer and the ^{13}C -labelled phenacetin. The microsomes were to be added last as a precaution against damage from prolonged exposure to the excess acetaldehyde to be added. As shown in Table 2, ca. 3 μmoles of ^{13}C -labelled phenacetin and 100 μmoles of glucose-6-phosphate were being used in the 1.5 ml of in vitro solution. Since this quantity of glucose-6-phosphate gives very strong resonances, it was decided

to add 72 μ moles of acetaldehyde because this amount would give strong spectral resonances and would be a ca. 24-fold excess over the phenacetin. This amount should be sufficient to upset the equilibria.

When all components except the microsomes had been placed in the NMR tube (in accordance with Section D of Chapter 2), 3.17 μ g of cold (2°C) acetaldehyde was weighed directly into the solution by use of a cold dropper held just above the solution in the NMR tube. The antivortex plug was quickly added and the tube stoppered. The spectrum of this mixture was rapidly accumulated in the next two hours (acquisition time of 0.3 s) and is shown as Figure 22 (this figure and its assignments are nearly identical to that of Figure 13 obtained in the first experiment). Despite the poor S/N, the methine carbon of the gem-diol form of acetaldehyde is just visible at $\delta = 89.52$ while that of the native form is not visible at $\delta = 208.12$. Together, the areas of these two resonances should be ca. 70% (from 72/100) of the area of one carbon of glucose-6-phosphate. Its C-1 resonances at $\delta = 97.10$ and $\delta = 93.30$ are about nine times as intense as the lone adjacent acetaldehyde resonance. To test for the vaporized acetaldehyde above the plug, the TLC detection of its 2,4-dinitrophenylhydrazone derivative

Figure 22. ^{13}C NMR spectrum of in vitro reaction mixture #4 prior to addition of microsomes.

was carried out (detailed in Section D of this chapter). The 2,4-dinitrophenylhydrazine solution turned cloudy after only a few syringe volumes of gas had been removed from the air space. The TLC plates showed distinct, intense spots for the acetaldehyde derivative and the 2,4-dinitrophenylhydrazine.

Together, the resonance intensities and the TLC observations provide evidence for the very rapid vaporization of acetaldehyde out of solution. Future attempts to weigh in the acetaldehyde were abandoned. The only method used to check on the amount added would be height of the acetaldehyde resonances. Since this is not quantitative, the control of the amount added would be by trial and error. It was desirable to have enough to observe and to reverse the equilibria, but not so much that the hemiacetal would not be seen due to dynamic range problems or damage to the metabolic system.

The results of Figure 22 suggested that more acetaldehyde should be added to the mixture to replace that lost by vaporization. Immediately prior to addition of the 50 mg of microsomal protein, 3 drops of chilled acetaldehyde were added via a chilled disposable pipet. The microsomes were added, the solution quickly homogenized by gentle vortex mixing, the antivortex plug positioned, and the tube sealed. The spectrum after

9.3 hours of accumulation is shown in Figure 23.

In this ^{13}C spectrum the methine and carbonyl resonances from the two forms of acetaldehyde are clearly visible at $\delta = 89.32$ and $\delta = 208.12$. Together their areas approximately equal the combined area of the two resonances of C-1 of glucose-6-phosphate*. Thus the quantity of acetaldehyde remaining in solution -- much has vaporized into the air space above the plug -- is in the range of the 24-fold excess whose original addition was attempted by weighing. In total, a much larger amount must have been added.

The most striking feature of Figure 23 is the appearance of the narrow, low-intensity resonance at $\delta = 95.84$. This is centered in the two ppm region, $\delta = 94.57$ - 96.50 , predicted for the ^{13}C -labelled hemiacetal resonance (Section E of this chapter). Besides occurring in the correct chemical shift range, it meets other criteria, detailed in Section E, necessary for identification as due to a hemiacetal intermediate.

Firstly, the resonance is narrow and has a line width

* The difference in T_1 's of the acetaldehyde and glucose-6-phosphate carbons is recognized, and because of this no quantitative conclusion is drawn regarding the number of μmoles of acetaldehyde added.

Figure 23. ^{13}C NMR spectrum of in vitro reaction mixture #4 after 9.3 hours.

comparable to those of the phenacetin and glucose-6-phosphate. Secondly, the intensity is not greater than that of the ^{13}C -labelled phenacetin prior to the start of metabolism (see Figure 22). Unfortunately, one of the most important proofs, the observation of a doublet in an off-resonance decoupling experiment, was not obtained. However, with the excellent agreement between observed and predicted chemical shifts and the intensity and line width arguments, a degree of confidence can be expressed in $\delta = 95.84$ as that of the hemiacetal. Hopefully, a greater concentration would be achieved so that an off-resonance proton decoupled spectrum would give good S/N and verify that $\delta = 95.84$ becomes a doublet. There is only one other possibility for the origin of $\delta = 95.84$, and that is that it originated from a metabolic transformation of the excess acetaldehyde and not the ^{13}C -labelled phenacetin. Further experiments were required to verify the nature of this resonance and assure that it is that of the hemiacetal.

There are other important features to observe in Figure 23. It is interesting to note that in this spectrum the methyl resonances from the two forms of acetaldehyde, $\delta = 31.27$ for the native form and $\delta = 24.33$ for the gem-diol form, are almost completely absent. This is in direct contrast to that observed for acetaldehyde

in water and the D₂O buffer. The spectra of these systems, already included as Figure 11.A and 11.B, clearly show the methyl carbon of each form as having a more intense resonance (50-60% greater) than that of their corresponding methine resonances. However, as with the relative areas of $\delta = 208.12$ and $\delta = 89.32$ in Figure 11, these areas in Figure 23 indicate that a similar equilibrium between acetaldehyde and the gem-diol has been set up in the in vitro mixture. Originally upon observing the methyl intensities in Figure 23, it was speculated that the methine carbons had become so much more intense due to the ¹³C-label ending up on the acetaldehyde and its gem-diol -- the direct proof sought for confirmation of the functioning of the assembled metabolic mixture. This reasoning will not be pursued here as the results of the next experiment¹ (Section H, vide infra) contradict this conclusion and proof. A complete analysis will be included there.

Another feature to note in Figure 23 is the resonance intensity of the residual ¹³C-labelled phenacetin at $\delta = 65.79$. As in previous experiments, the decrease in its intensity between Figures 22 and 23 is clear indication of a chemical reaction occurring at this labelled methylene carbon. In this experiment however, the observed decrease is not as large as noted

in experiments #1 and #3. In experiment #1, the same system contained less microsomal protein (40 mg as opposed to the 50 mg used in experiment #4) but led to near complete disappearance of the labelled phenacetin from the spectra*. This is observed by comparing Figures 13 and 15.A. Likewise in experiment #3, the spectra taken after assembly of the same mixture except with 50 mg of microsomal protein, Figure 20.A and 20.B, display a phenacetin resonance less than that observed in Figure 23.

The larger quantity of non-metabolized phenacetin in experiment #4 can be explained as an effect of the presence of the excess acetaldehyde. In the complete equilibria shown in Figure 21, a question mark was placed by the reversibility of the phenacetin to hemiacetal reaction. Due to the enzymic catalysts of this reaction and the fact that Garland determined it to be the rate determining step, there was doubt as to whether the excess acetaldehyde could effectively prevent or

*Since the metabolism has been noted to be very slow after the original rapid rate, the fact that Figure 15.A was taken at a later time in the experiment (28-35.5 hours) than was Figure 23 (0-9.3 hours) would not lead to the large difference noted between the two. Such an argument is reinforced by the similarity of the phenacetin resonance in Figure 20.A and 20.B, the rapid (0-1.5 hours) and lengthy (1.5-24 hours) spectra of the metabolic mixture of experiment #3 (see Section F).

reverse phenacetin metabolism. The relative increase in the amount of phenacetin noted in Figure 23 indicates that there has been a minor effect at this step. The only other possible explanation for the intensity of the phenacetin resonance is the loss of enzymic activity due to aging of the microsomes either in the experiment or in storage after original isolation. This will be ruled out by the one final manipulation carried out on this experiment mixture.

Thus, the addition of the excess acetaldehyde has had considerable effect on the chemical equilibria, as readily observed in Figure 23. The question which arose was: would the closed metabolic system respond in the expected manner to the removal of some of this acetaldehyde and the establishment of new equilibria? The method chosen to achieve this was to remove the cork stopper and leave the NMR tube open for ca. 30 minutes. During this time a distinct odor of acetaldehyde was noticeable in the work area. After this time period, the NMR tube was resealed and allowed to sit for ca. 1 hour to re-equilibrate. The ^{13}C NMR spectrum was then taken, Figure 24.

Upon examination of this spectrum, it is obvious that the manipulation has resulted in a decrease in the amount of excess acetaldehyde present in the metabolic

Figure 24. ^{13}C NMR spectrum of in vitro reaction mixture #4.
after loss of acetaldehyde.

mixture. The four acetaldehyde resonances have all decreased from the intensities observed in Figure 23. The two methyl carbons, $\delta = 31.27$ and $\delta = 24.33$, and the carbonyl carbon, $\delta = 208.12$, are not even visible in Figure 24. The methine carbon of the gem-diol form, $\delta = 89.32$, is about half the height of the two C-1 resonances of glucose-6-phosphate. In the original equilibrium (that shown in Figure 23), this acetaldehyde resonance had been ca. 40% larger than the two glucose-6-phosphate ones.

This decrease of excess acetaldehyde in the metabolic reaction mixture of experiment #4 should lead to a return to the results observed in the first three experiments -- complete and rapid metabolism of phenacetin with no trace of the hemiacetal nor of the final labelled acetaldehyde. Figure 24 shows that such changes have taken place. Firstly, the ^{13}C -labelled phenacetin resonance at $\delta = 65.79$ has decreased in intensity compared to that originally observed in Figure 23. This resumption of the phenacetin metabolism indicates that the microsomes have not suffered a loss of enzymic activity due to aging, a question which arose during discussion of the results observed in Figure 23, vide supra. The second important feature of Figure 24 is that the resonance which may be that of the hemiacetal, $\delta = 95.84$, is no longer present.

This absence is a very positive occurrence in that it indicates that this carbon is part of a molecule involved in the equilibria of this metabolic system. As the amount of excess acetaldehyde decreases there will be increased favoring of the final products, acetaldehyde and acetamidophen, over that of the hemiacetal intermediate.

The predicted and observed decrease of $\delta = 95.84$ in Figure 24 thus becomes one part of the evidence that this resonance is that of the hemiacetal intermediate (one must still prove that this resonance does not arise from the acetaldehyde).

These results from experiment #4 represented the first NMR evidence for the existence of the hemiacetal. While the resonance at $\delta = 95.84$ was promising, there was a need to repeat its spectral appearance and, hopefully, to intensify it so that further NMR experiments could be attempted. It was the results of Figure 24 which suggested the approach which would be used in experiment #5, the subject of Section H.

H. Results of In Vitro Experiment #5

As observed in the last part of experiment #4, the $\delta = 95.84$ resonance intensity proved to be sensitive to the amount of excess acetaldehyde in the solution. When the amount of acetaldehyde was decreased, the

resonance intensity decreased. For in vitro experiment #5, the opposite would be attempted. The amount of excess acetaldehyde would be increased and this, hopefully, would lead to a reciprocal increase in the amount of the species represented by $\delta = 95.84$. A second problem to be dealt with in this experiment was the reversal of the resonance intensities of the methyl and methine carbons of the native and gem-diol forms of acetaldehyde -- compare those in the buffer solution, Figure 11.B, with those in an in vitro mixture, Figure 23. The last ambiguity that this experiment must handle is the origin of the $\delta = 95.84$ resonance: does it arise from the metabolism of phenacetin or from acetaldehyde?

The last two problems can be solved by carefully choosing the order in which the in vitro reaction mixture is assembled and at which points the spectra are taken. For experiment #5, the original reaction mixture contained the three components of the NADPH-generating system, $MgCl_2$, EDTA and the microsomal protein. All were present in the phosphate D_2O buffer in the amounts indicated in Table 2, and their assembly was carried out according to the method in Section D in Chapter 2. The spectrum of this mixture is shown as Figure 25. It corresponds very closely to Figure 19, the spectrum in experiment #3 of the same mixture. In both spectra, all the resonances

Figure 25. ^{13}C NMR spectrum of in vitro reaction mixture #5 prior to addition of phenacetin and acetaldehyde.

can and have been identified as belonging to the unreacted starting components.

The NMR tube was now opened, the plug removed, and 4 drops of chilled acetaldehyde added to the reaction mixture. After gently and rapidly vortexing this solution, the antivortex plug was re-inserted. Quickly 1 drop of chilled acetaldehyde was dropped on top of the plug and the tube immediately corked and sealed with Parafilm. This last drop of acetaldehyde was added to directly saturate the air space above the plug and leave more acetaldehyde in the aqueous solution. The ^{13}C NMR spectrum was acquired over the next 13 hours and is shown as Figure 26.

The resonances of acetaldehyde are readily observed in this spectrum. The methine carbon resonance of the gem-diol form of acetaldehyde, $\delta = 89.32$, is approximately twice as intense as any glucose-6-phosphate resonance, and the acetaldehyde carbonyl, $\delta = 208.12$, is about equal to the glucose-6-phosphate C-1 resonances. Similar comparisons of these acetaldehyde and glucose-6-phosphate resonances in experiment #4 (Figure 23) show the acetaldehyde intensities to be about one half as large. Thus, in experiment #5 the amount of excess acetaldehyde in solution has been ca. doubled when compared to that in experiment #4. Hopefully this will

Figure 26. ^{13}C NMR spectrum of in vitro
reaction mixture #5 after
addition of acetaldehyde but
prior to phenacetin addition.

be sufficient to increase the intensity of $\delta = 95.84$.

Due to the amount of acetaldehyde present in the aqueous environment the methyl resonances of acetaldehyde are visible for the first time. When Figure 23 of experiment #4 was first analyzed, it was noted that the methine resonances were much more intense than the methyl ones, a situation opposite to what was observed for acetaldehyde in buffer (Figure 11.B). One possible explanation for this reversal was that the two methine resonances had been enhanced by the production of ^{13}C -labelled acetaldehyde from the phenacetin metabolism which had taken place in the experiment. The results of Figure 26 from the current experiment eliminate this as a possible explanation. Here, no ^{13}C -labelled phenacetin has been added and no metabolism has occurred to produce labelled acetaldehyde. Thus, in the in vitro metabolic reaction mixture assembled, the acetaldehyde resonances have opposite relative intensities to those observed from an aqueous solution. No labelled acetaldehyde was observed in Figure 23 from experiment #4, just the natural spectrum of acetaldehyde in the in vitro metabolic solution. Only from a very careful quantitative study of the relative areas of all of the acetaldehyde resonances in spectra taken immediately prior to and after phenacetin addition (e.g. Figures 26 and 27) would it be possible to

conclude that ^{13}C -labelled phenacetin is contributing to $\delta = 208.12$ and $\delta = 89.32$ via metabolism. No such study was carried out.

The reason for this reversal of relative intensities of acetaldehyde is seated in changes in the relaxation times of the carbons (identical spectrometer parameters were used to accumulate and record Figures 11 and 26). It is likely that the acetaldehyde has become involved in non-covalent molecular associations with various system components -- both native and gem-diol molecules are small and have labile centers in the carbonyl and dihydroxy carbons, respectively. There is evidence for such interactions in that the acetaldehyde methyl resonances in Figure 26 are not sharp singular peaks at $\delta = 31.27$ and $\delta = 24.33$. For the gem-diol form there are at least three distinct sharp resonances at $\delta = 25.11$, $\delta = 24.33$ and $\delta = 23.14$. It is likely that Van der Waals, ionic or hydrogen bonding interactions are occurring between the gem-diol molecule and the various environments found in the microsomal protein. Such interactions have altered the acetaldehyde's molecular dynamics and led to increased T_1 's for the methyl carbons relative to those of the methine carbons. Under the pulse conditions used to obtain the spectra, the increased T_1 's give a decreased intensity due to

incomplete recovery of the magnetization between pulses.

Figure 26 can also be used to answer the very important question as to the origin of $\delta = 95.84$, the resonance which may be that of the hemiacetal. Due to the order in which the in vitro mixture of experiment #4 was assembled, the $\delta = 95.84$ resonance first appeared in the same spectra in which the excess acetaldehyde was first observed (Figure 23). Thus, it was not possible to rule out the possibility that $\delta = 95.84$ might be a product from some microsomal metabolism of acetaldehyde rather than phenacetin. In experiment #5, Figure 26 is the spectrum of the metabolic mixture with excess acetaldehyde but without phenacetin present. Now, there is no resonance observable in the region of $\delta = 95.8$, despite the fact that there is a larger amount of excess acetaldehyde available to the microsomes in this experiment than there was in experiment #4. There appears to be only one weak resonance at $\delta = 92.07$ which arises, as previously seen in Figure 11.A, from an impurity in the bottle of acetaldehyde. With the origin of $\delta = 95.84$ not being the acetaldehyde, the appearance of this peak after phenacetin addition to the experiment #5 mixture will prove that it arises from the ^{13}C -labelled phenacetin.

Working quickly to minimize loss of acetaldehyde, the in vitro reaction mixture of experiment #5 was

next opened, the antivortex plug removed and the 3.14 μ mole of ^{13}C -labelled phenacetin added. Next, O_2 was gently bubbled in for ca. 2 seconds, the plug repositioned and the NMR tube corked and covered with Parafilm. The entire operation was completed in ca. 25 seconds. The spectrum of this complete in vitro metabolic system is shown in Figure 27. Comparing the intensities of the acetaldehyde resonances in Figures 26 and 27, one can estimate that ca. 20% of the excess acetaldehyde in the solution has been lost during the addition of the phenacetin.

Due to the small residual intensity for the ^{13}C -labelled phenacetin at $\delta = 65.79$, two conclusions can be drawn. First, the metabolic machinery is operative -- instead of being larger than all of the glucose-6-phosphate resonances, the phenacetin is smaller. Second, the excess acetaldehyde has again established an equilibrium which prevents the complete metabolism of the phenacetin.

With the metabolism operative and the excess acetaldehyde creating conditions favorable for observation of the hemiacetal, the resonance at $\delta = 95.84$ is observed for the second time. The doubling of the amount of excess acetaldehyde from experiment #4 to experiment #5, has likewise doubled the intensity of this resonance.

Figure 27. ^{13}C NMR spectrum of in vitro reaction mixture #5 after addition of acetaldehyde and phenacetin.

Therefore, in addition to the items pointed out during the discussion of this resonance in experiment #4, there are now two more articles which serve as proof for this being the hemiacetal resonance. First, the resonance at $\delta = 95.84$ only appeared after addition of the ^{13}C -labelled phenacetin, and thus its origin is most certainly from the phenacetin. The second proof is the increase in the intensity of the $\delta = 95.84$ resonance with the increased amount of excess acetaldehyde. This implies that the carbon belonging to the resonance is part of a molecule involved in the equilibria between the starting substrate phenacetin and the end products (these are equilibria acetaldehyde can affect). Appropriately, the $\delta = 95.84$ intensity is not greater than that given by 3.14 μmole of unmetabolized, ^{13}C -labelled phenacetin (observed to be greater than the glucose-6-phosphate resonances, Section D).

Having obtained an enhanced intensity for the $\delta=95.84$ resonance, it was now possible to perform the off-resonance proton decoupling experiment on the reaction mixture. This spectrum was accumulated in nine hours immediately following completion of the preceding run (Figure 27) in which the $\delta = 95.84$ resonance was observed. The off-resonance decoupled spectrum is shown in Figure 28, along with an expansion of the $\delta = 88-98$ region. The

Figure 28. ^{13}C off-resonance decoupling spectrum of in vitro reaction mixture #5.

eight resonances observed in this 10 ppm range are listed and analyzed in Table 3. In assigning these ten resonances, the doublets expected for the two C-1 methine carbons of glucose-6-phosphate and the one doublet for the methine carbon of the gem-diol form of acetaldehyde were first identified. There remained only one doublet, which as shown in Table 3, was centered at $\delta = 95.83$. Therefore, the $\delta = 95.84$ resonance was immediately identified as a carbon having only one directly bonded hydrogen atom. This is one of the important experimental results needed for the verification of this resonance as belonging to the ^{13}C -labelled hemiacetal intermediate.

The results obtained from the spectra of experiment #5, Figures 25 to 28, offer proof that the $\delta = 95.84$ resonance belongs to the hemiacetal intermediate created during phenacetin metabolism. The most important of these results were as follows.

Firstly, the $\delta = 95.84$ resonance was shown to arise from the ^{13}C -labelled phenacetin and not from the acetaldehyde. Secondly, the amount of excess acetaldehyde in solution affected the intensity of $\delta = 95.84$. This result implies that this carbon was located on a molecular species involved in the equilibria affected by the acetaldehyde, that is, the equilibria of the metabolism of phenacetin to acetaldehyde and acetamidophen.

Table 3

The theoretical and observed results from the off-resonance decoupling experiment carried out in experiment #5.

Compound	Carbon	δ in proton decoupled experiment	Carbon Type	Expected multiplicity in off-reson- ance decoupling experiment	δ of multiplets observed in off- resonance decoupling	Center of multiplets observed in off-resonance decoupling experiment
Glucose- 6-phosphate	C-1, β	97.10	CH	doublet	97.79	97.09
	C-1, α	93.30	CH	doublet	96.39	
Acetaldehyde					94.07	93.26
	gem-diol	89.32	CH	doublet	92.46	
					90.09	89.30
?	?	95.84	?	doublet	88.51	
					96.59	95.83
					95.07	

Thirdly, the $\delta = 95.84$ carbon has only one directly-bonded hydrogen. These results, in conjunction with those from previous experiments, calculations and considerations, present a very conclusive case for the identification of $\delta = 95.84$ as that of the labelled hemiacetal intermediate produced during the metabolism of ^{13}C -labelled phenacetin by rat liver microsomes.

I. Results of In Vitro Experiment #6

While the evidence uncovered in the previous experiments indicated $\delta = 95.84$ was the hemiacetal resonance, there remained the possibility that this resonance was somehow peculiar to the two experiments, #4 and #5, in which it appeared. Since it was a very weak resonance in experiment #4, it was now desirable to be able to repeat the results of experiment #5. If $\delta = 95.84$ again appeared it would be a further proof for it belonging to the hemiacetal.

To prove that the results are truly repeatable, many of the metabolic components were made or obtained anew. Thus, new bottles of the reagents glucose-6-phosphate, NADP and EDTA were used. Also, a new and second preparation of the rat liver microsomes was carried out. These were used in experiment #6 within 24 hours after their final isolation and storage at -60°C .

It was also decided to put an even larger quantity of excess acetaldehyde into the metabolic mixture of experiment #6 than was present in experiment #5. Hopefully, this would further intensify the $\delta = 95.84$ resonance. The assembly of the metabolic mixture and the points at which spectra were taken were nearly identical to those in experiment #5. For this reason, no detailed discussion of the results of experiment #6 will be included here*.

The spectrum of the metabolic mixture in experiment #6 with the excess chilled acetaldehyde added but prior to the addition of ^{13}C -labelled phenacetin is shown in Figure 29 (the corresponding spectrum in experiment #5 is Figure 26). Three to four drops of acetaldehyde had been added into the solution and one onto the top of the antivortex plug. However, comparison of $\delta=89.32$ and $\delta=208.12$ in Figure 26 and 29 indicates that there is even less acetaldehyde in the reaction mixture of experiment #6 than there was in experiment #5. During the addition process there must have been a large loss due to vaporization resulting from contact with some source of heat or from failure to work quickly enough.

* For a detailed discussion of each spectrum, the reader is referred to the corresponding spectrum in experiment #5.

Figure 29. ^{13}C NMR spectrum of in vitro reaction mixture #6 after addition of acetaldehyde but prior to addition of phenacetin.

The one new resonance not previously observed is a very broad one centered at $\delta = 67$, the identity and origin of which are not known. The remainder of the spectrum, both resonance positions and intensities, are as expected.

The reaction mixture was next unsealed, the antivortex plug removed, and four more drops of chilled acetaldehyde added to increase the amount in solution. Immediately afterwards, the ^{13}C -labelled phenacetin was added, the plug resealed, 2 more drops of acetaldehyde added on top of it, and the NMR tube resealed securely. The spectrum of this mixture is shown in Figure 30 (the corresponding spectrum in experiment #5 is Figure 27). Now the resonances at $\delta = 208.12$ and $\delta = 89.32$ make it obvious that the amount of acetaldehyde in this solution had been increased to the desired level. There is also evidence, from $\delta = 65.79$, that a small quantity of phenacetin has remained unmetabolized. The unexplained broad resonance at $\delta = 67$ is not present in this spectrum, unless it is included in the phenacetin resonance, the base of which is broader than previously observed.

The most important feature of Figure 30 is the resonance at $\delta = 95.84$. Once again, it has appeared after phenacetin metabolism has taken place in the presence of excess acetaldehyde. Since the amount of this excess acetaldehyde is ca. 2-3 times greater than in experiment #5,

Figure 30. ^{13}C NMR spectrum of
in vitro reaction mixture #6
(with acetaldehyde and phen-
acetin added).

the amount of the carbon represented by $\delta = 95.84$ should also have increased due to the equilibria in experiment #6 being directed back even more towards the hemiacetal from the end products. Noting that the amount of glucose-6-phosphate is the same in experiments #5 and #6 and that identical spectra acquisition parameters were used, it was possible to measure the relative amounts of the $\delta = 95.84$ carbon in the two experiments. This was done by comparing the area of $\delta = 95.84$ with the area from one carbon of glucose-6-phosphate. The glucose-6-phosphate carbon chosen is C-1, for which there are two resonances; that from the α form, $\delta = 93.30$, and that of the β form, $\delta = 97.10$. Therefore, the ratio R can be defined as follows:

$$R = \frac{(\text{area of hemiacetal carbon})}{(\text{area of glucose-6-phosphate C-1 carbon})}$$

$$= \frac{(\text{integral of } \delta = 95.84)}{(\text{integral of } \delta = 93.30 + \text{integral of } \delta = 97.10)}$$

Thus, R should increase in going from experiment #5 to experiment #6 due to the larger excess of acetaldehyde in #6. Using the integrals of Figures 27 and 30, the calculation of R in experiments #5 and #6 is summarized below:

	Experiment #5 (Figure 27)	Experiment #6 (Figure 30)
Integral δ = 95.84	1.20	1.95
Integral δ = 93.30	1.30	1.25
Integral δ = 97.10	2.20	2.55
$\therefore R$	0.343	0.513

As expected then, the larger quantity of acetaldehyde in experiment #6 has caused there to be a greater amount of hemiacetal than in experiment #5. Of course, the amount of hemiacetal in a reaction mixture will not increase indefinitely as more acetaldehyde is added, but rather the final amount will be limited by the phenacetin added. In addition, more acetaldehyde in solution than what is observed in Figure 30 may lead to a dynamic range problem and loss of weaker resonances.

The last spectrum obtained from the reaction mixture of this experiment is one from an off-resonance decoupling experiment. This is included here as Figure 31 (the corresponding spectrum in experiment #5 is Figure 28).

Figure 31. ^{13}C off-resonance decoupling
spectrum of in vitro reaction
mixture #6.

Due to the decoupler being placed 2 ppm to lower field for Figure 31 than was used for Figure 28, the splittings in Figure 31 are slightly smaller. Upon analysis of the spectrum and as shown in the expanded plot of Figure 31, the resonance at $\delta = 95.84$ was once again found to give a doublet. Therefore, it is from a carbon with only one directly-bonded hydrogen.

Thus, the results of experiment #6, as given in Figures 29, 30 and 31, verify that the results of the previous experiments -- those used to observe and prove the existence of the hemiacetal intermediate -- are repeatable.

Chapter 4

SUMMARY AND CONCLUSION

A. The ^{13}C NMR Results Regarding the Oxidative Metabolism of Phenacetin

Due to the toxic effect of the widely used pharmaceutical phenacetin, many studies have been undertaken to determine its metabolic pathway. With results at the molecular level it was hoped that an understanding would develop of how phenacetin exerts its toxic effect on the kidneys. Early in these studies it was established that the first metabolic step occurred in the liver and involved oxidative deethylation of phenacetin to form acetamidophen and acetaldehyde. Preliminary work indirectly indicated that the first step of this reaction was formation of a hemiacetal intermediate. After a toxicity study implicated this step as being important, direct proof of the hemiacetal's existence became very desirable.

For his mass spectrometry studies Garland had ^{13}C -labelled the ethoxy methylene carbon of phenacetin. Thus, the obvious technique with which to try to observe the hemiacetal intermediate was ^{13}C NMR spectroscopy. The advantages to this approach were numerous. First, the ^{13}C chemical shift scale is very large and its sensitivity to molecular environments permits one to monitor even subtle

chemical events. Second, the additivity of chemical shifts permits fairly accurate prediction of values for new species by use of model compounds. Third, the ^{13}C label was located at the carbon of interest (that which would become the hemiacetal) in the phenacetin molecule. The 60% labelling of this carbon would allow work with smaller quantities and overcome the relative insensitivity of natural abundance ^{13}C NMR. Fourth, the technique is not harsh so as to perturb the in vitro system.

The entire metabolic system -- ^{13}C -labelled phenacetin, rat liver microsomal protein, EDTA, MgCl_2 , a phosphate pH 7.4 buffer and an NADPH-generating system consisting of NADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase -- was assembled directly in the NMR tube. After spectral identification of the various components and proof that phenacetin was in fact being metabolized, the search for the hemiacetal was begun. After excess acetaldehyde had been added to the in vitro mixture to alter the equilibria to favor the stopping of the metabolic reaction at the intermediate, a resonance at $\delta = 95.84$ was observed. The identification of this resonance as that of the ^{13}C -labelled hemiacetal was achieved by consideration of the following facts:

- (i) The ^{13}C -labelled phenacetin resonance at $\delta = 65.79$ disappears once metabolism begins

and, correspondingly, the $\delta = 95.84$ resonance appears. Thus, the origin of the carbon represented by the latter is from the ^{13}C -labelled ethoxy methylene carbon of phenacetin.

(ii) The resonance at $\delta = 95.84$ is a sharp narrow line representing one unique molecular environment.

(iii) The intensity of $\delta = 95.84$ does not become greater than that ($\delta = 65.79$) of the unmetabolized ^{13}C -labelled phenacetin. Had it become larger than this, the $\delta = 95.84$ carbon would have to have originated from something other than the phenacetin.

(iv) The $\delta = 95.84$ resonance is in excellent agreement with the predicted value for the hemiacetal carbon, $\delta = 94.57-96.50$. The model compound system used to make this prediction is closely related to the hemiacetal and thus the predicted values should be very accurate.

(v) The amount of excess acetaldehyde in solution effects the intensity of the $\delta = 95.84$ resonance in the manner predicted by Le Chatelier's principle. As the amount of acetaldehyde is increased, the equilibria between phenacetin and acetamidophen and acetaldehyde will be forced back further, and the lifetime and the intensity of the resonance of the hemiacetal should increase.

The carbon resonating at $\delta = 95.84$ behaves in this manner, and thus it is involved in the perturbed equilibria.

(vi) In an off-resonance proton decoupling experiment, the resonance at $\delta = 95.84$ gives a doublet. This verifies that the $\delta = 95.84$ carbon has only one directly bonded hydrogen as expected for the labelled hemiacetal carbon.

(vii) All the results of the experiments are repeatable, even when using new sources of the reaction components. Given all these facts then, the resonance at $\delta = 95.84$ can definitely be assigned to that of the hemiacetal intermediate. The ^{13}C NMR method has proven to be particularly suited to observe directly the hemiacetal intermediate in phenacetin metabolism.

B. Suggestions for Future Work

The ^{13}C NMR approach to the above problem is very appropriate. The single most important factor in its success is the fact that the phenacetin has been ^{13}C -labelled at the carbon of interest. Had natural abundance ^{13}C NMR been attempted it is very unlikely that there would have been sufficient sensitivity to observe the hemiacetal. There are definite limits as to how much material can be placed in a given volume. An in vitro reaction mixture

for natural abundance ^{13}C NMR of the phenacetin metabolism cannot be formed because of the large amounts of phenacetin and other components which would have to be placed in the ca. 1.5 ml of buffer.

Thus, in suggesting the following NMR studies on the metabolism of various pharmaceuticals, the primary concern given is to the availability of the labelled compound. Dr. Garland currently has or can easily label the following drugs. Given these, the suggestions for NMR investigations of the reaction mechanisms of their biotransformation are:

- (i) Phenacetin is known to undergo some N-deacetylation during its metabolism(1).

The following two ^{13}C -labelled analogues of phenacetin could be used to study this reaction mechanism.

Much the same maneuver with the excess acetaldehyde could be used to force the equilibria towards any intermediates. The one problem would be to find an enzyme system which would permit the above metabolism but prevent the deethylation metabolism. If both are occurring the deethylation would dominate and obscure the resonances of interest from the N-deacetylation.

- (ii) One report has already appeared on the use of ^{13}C NMR for the detection and identification of metabolites of ^{13}C -labelled Amitriptyline in dog urine after the drug had been orally administered(37). The antidepressant Amitriptyline is known to undergo the following metabolism in man, rat and rabbit liver microsomes.

Once again, one of the final products, formaldehyde, would be used in an in vitro experiment to alter the equilibria to favor the intermediate.

Also available is the ^{15}N -labelled analogue of Amitriptyline. In this case one would use ^{15}N NMR, add excess formaldehyde, and hopefully observe the intermediate. If the ^{15}N and ^{13}C labels were both present on the same molecule, the ^{13}C NMR would give the same results as previous except that some lines would be split due to the ^{15}N - ^{13}C coupling.

- (iii) The liver microsomes of man, rat and dog are known to cause the following metabolism of diazepam, or Valium, an antianxiety drug.

The use of ^{13}C NMR and the addition of excess formaldehyde should be sufficient to verify the existence of the proposed intermediate.

- (iv) The hypnotic drug Dalmane is also metabolized by man, rat and dog liver microsomes.

By adding excess acetaldehyde and carrying out a ^{13}C NMR investigation, there are good chances to observe the intermediate if it exists.

- (v) There is one metabolic system which is best studied using ^{15}N NMR. This is the metabolism of Clonazepam, not in microsomes, but rather with the soluble enzyme systems found in the cytosol of man, rat and dog. The metabolism is thought to proceed as follows:

This system would be much harder to study because there is no readily available end product which could be used for altering the equilibria towards the intermediates. Lowering the temperature might be one way of slowing the reaction down to increase the lifetime of the intermediates.

For all of these suggested experiments, a good approach will probably turn out to be one similar to that used to observe the short-lived intermediate of phenacetin. This has been meticulously documented in Part II of this thesis, and, briefly, proposes that one examine the individual components of the system, predict the chemical shifts, and then proceed with the experimentation, altering the equilibria as necessary, to obtain as much spectral evidence as possible for the proposed intermediate.

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Appendix I

INTERPRETATION OF THE ^{13}C SPIN LATTICE RELAXATION TIMES OF INSECT PHEROMONES IN TERMS OF MOLECULAR AND INTRAMOLECULAR MOTIONS

A. Project Overview

A pheromone is a chemical or a mixture of chemicals that is secreted to the exterior by an organism and that causes one or more specific reactions in a receiving organism of the same species. Pheromones are known to elicit a variety of behavioral responses: locomotory stimulation, feeding, mating or ovipositional stimulation or deterrent, grooming, social coherence, alarm, aggression, etc. Isolation and identification of the first pheromone, the sex attractant of the female silkworm, was reported in 1959 (1). Due to their ability to attract and their possible use in manipulating the behavior of pests, sex pheromones have been the most extensively studied. By 1976 over 40 had been identified and these accounted for over half of the total pheromone literature. The chemical structures of these compounds have been very similar: generally an unbranched C-10 to C-18 chain with one or two points of unsaturation and usually terminating with a functional group such as acetate, aldehyde, alcohol or acid.

For the purpose of synthesizing and testing the activity of derivatives of pheromones, nine commercially available sex pheromones had been purchased by Dr. H.H. Mantsch. As part of the spectroscopic identification of any derivative, the ^{13}C NMR spectra of the starting material and the final product were to be obtained and analyzed by myself. The subjects of this appendix are, then, the NMR work that was performed in support of this project and the subsequent NMR work which was initiated after the synthesis work was aborted. This study is being reported in an appendix because not all aspects have been completed and because I do not have a sufficient background in some of the theoretical aspects used in the analysis of the relaxation data.

Section B of this appendix deals with the ^{13}C chemical shift assignments of the nine commercial sex pheromones. Having made these assignments, the spin-lattice relaxation times, T_1 , of the carbons are also given in this section. Section C contains the theory and the method by which the T_1 data is analyzed to estimate the overall anisotropic molecular motion and the internal motion about the carbon-carbon bonds. The results from such an analysis on three insect pheromones are presented in Sections D and E. A further discussion of the limitations and problems associated with this method of

analysis are discussed in Section F.

B. The ^{13}C Chemical Shifts and T_1 's of Nine Insect Sex Pheromones

The nine pheromones which were analyzed are listed in Table 1, along with the systematic species names and the common names of some of the insects with which they are associated (2). The structures and the IUPAC carbon numbering of the compounds are shown in Figure 1. Also included on the structures of the first eight pheromones is a three dimensional co-ordinate system defined by the three mutually perpendicular axes x, y and z. These relate to the T_1 analysis and will be explained in Section C.

The ^{13}C chemical shifts of the nine pheromones in CDCl_3 were obtained at 25.16 MHz on a Varian XL-100-15 spectrometer. For each compound, two or three separate runs were made, all of which used the maximum 8K data points for maximum resolution. The first utilized a sweep width of 5500 Hz so that all resonances could be observed. The second spectrum had a much narrower sweep width, 1000-2000 Hz, and was centered on the $\delta' = 10-65$ range so as to resolve as many of the bulk methylene carbons ($\delta = 29.7$) as possible. To aid in the assignments for compounds 2-5 and 9, a third experiment employing

Table 1. Insect sex attractant pheromones studied

No.	Pheromones		Insect	
	Systematic Name ("Common Name")	Systematic Name	Systematic Name	Common Name
1	Undecanal	Galleria mellonella		greater waxmoth
2	cis-7-dodecen-1-ol acetate ("Looperlure")	Trichoplusia ni Pseudoplusia includens		cabbage looper moth soybean looper moth
3	cis-7-hexadecen-1-ol acetate ("Hexalure")	Pectinophora gossypiella		pink bollworm moth
4	cis-8-dodecen-1-ol acetate	Grapholitha molesta		oriental fruit moth
5	trans-8-dodecen-1-ol acetate	Grapholitha molesta		oriental fruit moth
6	cis-11-tetradecen-1-ol	Platynota stultana Ostrinia nubilalis		leafroller moth European cornborer moth
7	trans-11-tetradecen-1-ol	Platynota stultana Platynota idaeusalis		leafroller moth tufted apple bud moth
8	cis-9-tricosene ("Muscalure")	Musca domestica		common housefly
9	2-methyl-cis-7,8-epoxy- octadecane ("Displaure")	Porthetria dispar		gypsy moth

Figure 1. The structures and carbon numbering for the pheromones of Table 1.

Figure 1. (continued)

Figure 1. (continued)

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off-resonance proton decoupling was run using the same sweep width as for the second spectrum of each compound.

The ^{13}C chemical shifts of the nine pheromones are given in Table 2. The reasoning behind each assignment will not be given. Rather, only the variety of techniques used will be described and some examples given.

By virtue of their isolation in characteristically different chemical shift regions, the following carbons are readily recognized: (i) the single carbonyl carbon in 1-5 ($\delta = 171-203$); (ii) the olefinic carbons of 2-8 ($\delta = 129.4-132.0$); (iii) the C-1 methylene carbons which are directly bonded to the oxygen atom in 2-7 ($\delta = 63.1-64.7$); and (iv) the C-7,8 methine carbons of the oxirane ring in 9.

Using the results of the off-resonance decoupling experiment further assignments were made. The methyl carbons in all compounds are readily distinguished from the methylene carbons. For compounds 2-5 where there are two different methyl resonances (i.e., one acetyl methyl and one terminal methyl on the paraffinic chain), the off-resonance decoupled spectrum distinguishes between the two by virtue of line width. It was expected and observed that the line widths of the quartet for acetyl methyl carbons would be very small since there are

TABLE 2
¹³C Chemical Shifts for 1-9 (from TMS internal)

Position	1	2	3	4	5	6	7	8	9
C-1	202.88	64.66	64.68	64.67	64.48	63.10	63.10	14.19	22.67
C-2	43.98	28.68	28.70	28.70	28.70	32.90	32.89	22.80	27.40
C-3	22.18	25.91	25.93	25.98	25.96	25.84	25.84	32.04	39.02
C-4	29.61	28.95	28.98	29.22	29.06	29.64*	29.69*	29.44	39.02*
C-5	29.36	27.15	29.69	29.22	29.06	29.64*	29.64*	29.66	26.96*
C-6	29.42	27.15	27.18	29.74	29.61	29.53*	29.60*	29.44	27.98
C-7	29.50	129.68	129.68	27.24	32.61	29.53*	29.53*	29.90	57.29
C-8	29.25	130.11	130.22	129.81	130.31	29.37	29.26	27.33	57.29
C-9	31.96	26.99	27.32	129.99	130.49	29.86	29.74	129.99	27.98
C-10	22.73	32.03	29.85	29.38	34.77	27.18	32.65	129.99	26.69
C-11	14.12	22.40	29.40	22.96	22.81	129.44	129.46	27.33	29.65
C-12		14.02	29.61	13.84	13.68	131.61	131.96	29.90	29.65
C-13			29.40			20.59	25.68	29.66*	29.65
C-14			31.99			14.45	14.07	29.69	29.65
C-15			22.76					29.69*	29.41
C-16			14.15					29.82	31.09
C-17								29.82	22.76
C-18								29.82	14.16
C-19								29.82	
C-20								29.40	
C-21								32.04	
C-22								22.80	
C-23								14.19	
Acetyl C=O		171.17	171.23	171.23	171.23				
Acetyl CH ₃		21.01	21.03	21.02	21.02				
C-2 Methyl									22.67

* Assignment uncertain, values may be interchanged.

no long range ^{13}C - ^1H couplings possible. Since the terminal methyls of the paraffinic chain in 2-5 are bonded to methylene groups, a fine structure (i.e. increased line width) is observed for each line of the methyl quartet in the off-resonance decoupled spectrum. Once the two methyl resonances in 9 have been identified from the off-resonance decoupling experiment, the assignment is made on the basis of the expected and observed 2:1 intensity relationship. For all these compounds displaying two methyl resonances (2-5 and 9), model compounds lead to the same assignments as obtained above.

The assignment of the individual olefinic carbons and the remaining methylene carbons was done solely on the basis of model compounds. Using tables from Stothers book (3), some methylene carbons can be assigned due to their proximity (i.e. α , β , γ) to a terminal methyl, carbonyl, acetyl, hydroxyl or olefinic functional group. However, it becomes much more difficult to assign those methylene carbons which are removed from functional groups and find themselves surrounded by other methylenes. For example, in 6 the six resonances for C-4 to C-9 are observed but they are within a range of 0.49 ppm. Fortunately, there was an excellent series of model compounds available to

complete these assignments and those of the olefinic carbons.

As part of his doctoral dissertation, J.G. Batchelor completed the chemical shift assignments of over 40 saturated, unsaturated, polyunsaturated and cyclopropane fatty acids (4,5). The values were reported to two significant figures after the decimal place, with the second figure used (as in Table 1) not as an absolute value but rather to demonstrate the proximity of the resonances. The certainty with which he could assign methylene and olefinic resonances was based on using lanthanide shift reagents and a ^{13}C -labelling technique. Using a mutant Escherichia coli which would metabolically incorporate sodium [1-90% ^{13}C]-acetate into its fatty acids, Batchelor was able to isolate fatty acids with their odd numbered carbons ^{13}C -labelled (6). Since his spectra and those of 1-9 were run under much the same conditions, there is an excellent agreement of resonance positions (i.e. to the second decimal place) for similar compounds. In this manner most of the remaining methylene and olefinic carbons are assigned for the pheromones. Only the C-4 and C-5 resonances of 9 are well separated, $\delta = 29.65$ and $\delta = 26.96$, and cannot be unambiguously assigned due to competing effects and poor model compounds.

Since these chemical shift assignments were made, the literature (7,8) has reported values for 2-5. In all cases there has been agreement between those in Table 1 and those determined by other workers.

Once the chemical shift assignments had been made, the T_1 of the carbons with directly-bonded hydrogen(s) were determined using the inversion recovery method (9). Due to the prohibitively long pulse delays necessary for determining the T_1 's of the slowly relaxing carbonyl carbons in 1-5, only that of 1 was measured using the homo-spoil method (10). All of the T_1 's measured are presented in Table 3. The standard deviation of each value was obtained and was typically of the order of ± 0.005 to ± 0.20 . There is a greater uncertainty for those carbons whose resonance came in a region in which many resonances were found. The analysis of the relaxation times is discussed in the next section.

TABLE 3
¹³C Spin-lattice Relaxation Times for 1-9 (sec)

Position	1	2	3	4	5	6	7	8	9
C-1	15.00 ^a	2.64	2.36	2.42	2.46	3.76	3.42	5.34	3.38
C-2	5.26	2.56	2.03	2.37	2.34	3.53	3.14	5.22	2.10
C-3	4.22	2.68	1.89	2.40	2.33	2.75	2.65	3.74	2.27
C-4	3.57	2.67	1.85	2.45	2.18	2.44	1.98	2.51	2.33*
C-5	3.71	2.43	2.08	2.45	2.18	2.44	1.98	1.84	1.89*
C-6	3.74	2.85	1.97	2.67	1.97	2.44	1.98	2.51	3.47
C-7	3.96	4.56	2.63	2.80	2.36	2.44	1.98	1.84	2.16
C-8	4.01	4.13	2.49	4.55	5.55	2.44	1.98	1.69	2.16
C-9	4.68	4.66	2.10	5.17	6.69	2.47	2.52	2.48	3.47
C-10	5.83	4.91	2.08	4.94	4.72	2.91	2.71	2.48	1.76
C-11	5.29	6.09	2.76	6.29	4.52	5.67	7.19	1.69	1.89
C-12		5.53	2.55	6.92	5.19	4.39	6.47	1.84	1.89
C-13			2.76			8.93	6.57	1.84	1.89
C-14			4.19			7.01	4.12	1.84	1.89
C-15			5.50					1.84	2.78
C-16			5.35					1.84	3.37
C-17								1.84	4.16
C-18								1.84	4.71
C-19								1.84	
C-20								2.51	
C-21								3.74	
C-22								5.22	
C-23								5.34	
Acetyl C=O									
Acetyl CH ₃		7.68	8.45	9.21	6.96				
C-2 Methyl									3.38

^aDetermined by homospoil method

* Depending on assignment; values may be inverted.

C. The Calculation of Molecular Motion From Dipolar Relaxation Times

The method of analysis of T_1 's to yield information on molecular motion is essentially that of Levine and co-workers (11-14). There are two types of motion to deal with: (i) overall anisotropic molecular motion, and (ii) the internal motion about each single bond.

The molecule is thought of as taking the shape of an axially symmetric prolate ellipsoid. The mutually perpendicular molecular axes are x , y , and z , with z defined as lying along the long axis. The molecular motion is described by D_z , D_x and D_y , the rotational diffusion coefficients about each axis. Due to the molecular shape, $D_z > D_x = D_y$, which is to say that the overall molecular motion is anisotropic (i.e. the molecule prefers to rotate about its long axis). In addition to and independent of these motions is the motion about each carbon-carbon single bond, described by the diffusion coefficient D_i for the i th bond*.

* Alternately, the motion could have been described in terms of the correlation times for each motion, τ_z , τ_x , τ_y and τ_i . The simple expression for relating the two is $\tau = 1/6D$.

The T_1 values for the molecules in solution will originate from dipole-dipole interactions. Under the extreme narrowing limit the well-known expression for the dipolar relaxation time, T_1^{DD} , of a nuclei reorienting in a magnetic field is (15):

$$\frac{1}{T_1} = \frac{1}{T_1^{DD}} = \frac{N \gamma_H^2 \gamma_C^2 \hbar^2}{10} \langle r^{-6} \rangle [J(\omega_H - \omega_C) + 3J(\omega_C) + 6J(\omega_H + \omega_C)]$$

Levine evaluated the spectral density functions, $J(\omega)$, using a stochastic rotational diffusion model and derived their dependence on the rotational diffusion coefficients. Briefly then, the method involves entering D_z , D_x , D_y and D_i and generating the dipolar relaxation times observed for the particular molecule. Dr. Levine kindly supplied a listing of the program he wrote to carry out these calculations. The following paragraphs briefly describe some of the approximations and subtleties involved in running the program.

It is important to recognize one of the key assumptions introduced in Levine's formulation. This states that the motion about each individual carbon-carbon bond is completely independent and uncorrelated with the motions about the other bonds. Also, each bond is free to rotate a full 360° , and all conformations,

from trans to fully eclipsed, occur with equal probability. These assumptions are somewhat contrary to the physical picture one would envision for linear alkanes in solution. For example, if a bond begins a trans to gauche jump, it may not be allowed to go to completion because it would introduce a large bend in the chain. However, if at the same time a bond β to the first were undergoing the same transition but in the opposite sense, both jumps might go to completion since their co-operative movement would only introduce a small kink in the chain while retaining its general linear nature. Levine's neglect of correlated rotations is a convenience for simplifying an already complex calculation, but is not so severe as to make this first approximation unreasonable.

Levine's calculation generates T_1 's away from the center of mass of the molecule. Thus, it is necessary to identify a particular carbon and generate the T_1 's towards each end of the molecule. The problem with identifying a center of mass is that it is constantly changing due to the internal motions. One must, then, choose a static conformation for this calculation. The most logical choice is with the molecule in the all-trans conformation, the one in which intramolecular physical interactions are minimized.

All the pheromone molecules are depicted in such a conformation in Figure 1. The center of mass of compounds 1-8 was determined with a revised version of QCPE-178 (for which I thank Dr. W.F. Murphy, NRCC), a program primarily designed to calculate the moments of inertia, I_x , I_y , and I_z , about the three principal molecular axes, x, y and z. Using the known bond lengths and angles, and arbitrarily choosing a reference point, the 3-D coordinates for each atom are entered into the program. The results are: a transposed frame of reference such that the three mutually perpendicular principal molecular axes are defined, a center of mass with coordinates (0,0,0) in the new system, the coordinates of all atoms in reference to the center of mass and principal axes, and the moments of inertia about the principal axes. The principal axes and center of mass for 1 to 8 are shown on Figure 1. A brief description where the center of mass sits, the moments of inertia I_x , I_y , and I_z , and the anisotropic ratio (vide supra) are given in Table 4.

Having designated a center of mass, it is necessary to enter the angles between the bonds about which rotation occurs. Levine had primarily investigated alkanes, for which all the angles are identical (109.8°). Since Levine's program was being utilized for many different types of molecules by multiple users at NRCC,

Table 4. Center of mass, moments of inertia and anisotropic ratio for 1-8

Compound No.	Center of Mass for static all-trans conformation	I_x amu·Å ²	I_y amu·Å ²	I_z amu·Å ²	Anisotropic Ratio $I_x (=I_y)/I_z$
<u>1</u>	-between C-5, C-6 on long axis	3255.0	3290.3	67.9	48.2
<u>2</u>	-between C-4, C-5 at C-4 level	6389.4	6595.4	238.4	27.2
<u>3</u>	-between C-6, C-7 (above C-6) - passes thru C-1, C-4, C-11	12490.9	12982.2	535.3	23.8
<u>4</u>	-between C-4, C-5 -near C-3, C-5, C-10	6458.1	6658.4	232.8	28.2
<u>5</u>	-between C-4, C-5 (near C-5) on long axis	7129.6	7212.6	115.5	62.1
<u>6</u>	-between C-6, C-7 (near C-7) -along long axis	6254.4	6344.0	124.6	50.5
<u>7</u>	-between C-6, C-7 (near C-7) -along long axis	6593.0	6641.4	83.4	79.3
<u>8</u>	-between C-12, C-13 (near C-12) -passes thru C-5 and between C-17, C-18	20462.7	21159.8	758.4	27.4

Dr. R. Somorjai modified the program to accomodate any angle (this entailed major revisions to the calculation of the second-order Wigner rotation matrix). For the first bond from the center of mass in an alkane the angle entered in the program is 35° . After this all single bonds are given a value of 109.5° , while 123.5° is used for double bonds.

The value of D_i that is entered is used for all carbon-carbon single bonds with the exception of the terminal $\text{CH}_2\text{-CH}_3$ bond (and possibly the previous $\text{CH}_2\text{-CH}_2$ bond). At the end of an alkane chain, the methyl group is known to undergo a much faster rotation. Thus, its D_i is better estimated from a separate calculation once the T_1 's for all carbons between it and the center of mass have been generated using one unique D_i value. For a double bond, a value of $D_i = (1) \cdot (10^{-10})$, i.e. no rotation, is used.

The program is set up to calculate T_1 's of methylene carbons where there are two directly-bonded hydrogens. The number of directly-bonded hydrogens enters into the formula for T_1 (vide infra) as N . Thus, to compare the computer generated T_1 's with the experimental ones it is necessary to multiply the experimental T_1 's for CH , CH_2 and CH_3 by $1/2$, 1 , and $3/2$, respectively. With this correction, all T_1 values are normalized and

can be directly compared to the program output. The normalized experimental T_1 values are entered into the program for whatever chain length one wishes. As a set of T_1 values is calculated for that chain (from the D_z , $D_x (=D_y)$ and D_i supplied), a calculation is undertaken to compare experimental and theoretical T_1 's. The "best-fit parameter", BFP, is defined as:

$$BFP = \sum_m^n |T_1^m \text{ exp} - T_1^m \text{ calc}|$$

m varies from 1 to n , the number of carbons in the chain.

The object is to minimize BFP and obtain the best fit between experiment and theory.

The last problem to be addressed deals with the range of D_z and D_x , D_y values. Levine defines the anisotropic ratio, $D_z/D_x (=D_y)$, to estimate the degree of anisotropic motion. Further, he assumes that the anisotropic ratio of any conformation is given by the ratio of the moments of inertia, $I_x (=I_y)/I_z$. When the molecule is in the all-trans conformation, the anisotropic ratio will be a maximum. It can vary down to 1, where the molecule would now behave isotropically (i.e. spherical shape). The maximum anisotropic ratio for compounds 1-8 are given in Table 4. It is expected that the molecule will continually change between various anisotropic states. The barrier to rotation about single bonds is only 3 kcal/mole (16).

However, at room temperature thermal or kinetic energy supplies ca. 15-20 kcal/mole, more than enough for the molecule to be truly dynamic.

For any further details of the actual theory or the program, the reader is referred to the original papers and the program. I have not made a concerted effort to understand fully all the details of Levine's theory and program. Rather, I only became acquainted with the assumptions and limitations and then applied the analysis to the T_1 data of the pheromones. Levine's modified program was supplied to me by Dr. Somorjai and my changes to it were: conversion from a terminal interactive program to a batch program; the ability to enter many sets of experimental T_1 data with varying chain lengths and to compare each set individually with the one calculated set of T_1 's; improved input and output with regards to the bond angles and D_i 's. The final program used is titled ROEVAM and is stored in Dr. Ian Smith's computer tapes at NRCC.

For running ROEVAM on batch, one first selects a certain number of anisotropic ratios covering the range from 1 up to the predicted maximum. A range of D_z values is chosen and the program calculates the corresponding $D_x = D_y$ for each value of D_z at each anisotropic ratio. A range of D_i 's is then entered along with the bond angles. The program then calculates the desired set of T_1 values using every possible combination of D_i , $D_x (=D_y)$ and D_z .

values. For each set of T_1 's, the best fit calculation is carried out to compare them to experimental values. Lastly, one looks for the smallest BFP at each anisotropic ratio and graphs and interprets the results. The next section deals with the results for the simplest pheromone studied, undecanal.

D. Molecular Motion of Undecanal, 1

The first work to quantify the molecular motion was carried out on undecanal, 1. There were two assumptions employed throughout the work. Firstly, the center of mass was located between C-5 and C-6 (its position in the all-trans conformation), regardless of the conformation it assumes. Secondly, the anisotropic ratio of overall molecular motion can vary between 1, the isotropic limit, and 48, the maximum from the ratio of the moments of inertia. Values of 1, 2, 5, 6, 7, 8, 10, 20, 30, 40 and 48 were actually used.

The T_1 's towards the methyl end of the molecule were analyzed first. The experimental values for C-6 to C-10, 3.74, 3.96, 4.01, 4.68, and 5.83 s. respectively, were generated using only one common D_i for the intervening bonds. At first, the values of $D_z, D_x (=D_y)$ and D_i were varied coarsely at each anisotropic ratio. Once the magnitude of each value was obvious, a much narrower range was run to determine more accurately the best

agreement between the theoretical and observed values. Figure 2 shows the relationship between the best-fit parameter and the anisotropic ratio. The respective values for D_z , $D_x (=D_y)$ and D_i which led to these BFP at each anisotropic ratio, are presented graphically in Figure 3.

As seen in Figure 2 the best fit over the entire anisotropic range occurs when the ratio of D_z to D_x is near 6. This is not an unreasonable result when one considers that on a time average the internal motions (D_i) take the molecule out of the all-trans conformation, i.e. one does not expect to see the all-trans linear shape all that often because of the many possibilities for internal bond rotations. A lower ratio is expected and observed, and it emphasizes the fact that in solution the molecule is dynamic and not fixed in the simple all-trans conformation as usually pictured.

The only way of improving the agreement between predicted and observed T_1 's at any anisotropic ratio would be to vary the D_i of each bond. Considering the 10-15% error in T_1 determination, it would not be very informative to get a perfect match between the theoretical and experimental T_1 's. The best-fit parameter for the anisotropic ratio of six is 1.23 (or on the average 0.25 per T_1). If one takes 10% of each experimental T_1 for C-6 to C-10 and sums these values, a reasonable error

Figure 2. Best-fit parameter as a function of the anisotropic ratio for fitting C-6 to C-10 of 1.

Figure 3. The rotational diffusion coefficients as a function of anisotropic ratio for fitting C-6 to C-10 of 1.

to expect for the five T_1 values would be ca. 2.2. All best-fit parameters over the entire anisotropic ratio range are less than this. The use of only one D_i is supported by this observation.

The values of the rotational diffusion coefficients at the anisotropic ratio of six are:

$$D_z = (6.11)(10^{10}), D_x = D_y = (1.03)(10^{10}) \text{ and } D_i = (1.12)(10^{10}).$$

The T_1 's generated for C-6 to C-10 were 3.74, 3.96, 4.68, 5.23 and 5.82 s, respectively. Using these D_z and $D_x = D_y$ for the overall motion and this D_i for C-6 to C-10, a separate D_i was determined to give the observed T_1 of the terminal C-11 methyl carbon. The value, D_w in Levine's rotation, was found to be $D_i = D_w = (3.77)(10^{10})$. As expected then the motion about this final single bond was much faster (3.4x) than the motion about the rest of the single bonds. A separate determination of D_w at each anisotropic ratio was not carried out but from observed trends, D_w will increase as the anisotropic ratio increases.

Attention was now turned to fitting the T_1 's for the other end of the molecule. A common D_i value was assumed for the bonds from C-5 to C-2, and again a special fit would be necessary for the single bond between C-2 and the terminal carbonyl C-1. A reasonable starting place was to use the values of D_z , $D_x (=D_y)$ and D_i found for an

anisotropic ratio of six for the other end of the molecule. The agreement between experimental and predicted values was good, giving a best-fit parameter of 0.90 for C-5 to C-2 (or 0.23 per carbon T_1). The experimental values were 3.71, 3.57, 4.22 and 5.26, while the generated ones were 3.74, 3.96, 4.68 and 5.23. A slight improvement could be effected by decreasing the D_i by 15% to $D_i = .(9.52)(10^9)$. As at the other end of the molecule, the terminal single bond was found to rotate faster than the other bonds in the chain. Due to the good agreement no comprehensive variance of D_z , D_x and D_i was carried out for this end of the molecule.

E. Molecular Motion of cis-11-tetradecen-1-ol, 6, and trans-11-tetradecen-1-ol, 7

Having studied a relatively simple molecule, the tetradecen-1-ols were chosen to check the motion predicted for cis and trans isomers. Further, 6 and 7 are both relatively linear, and unlike the other pair of isomers (4 and 5), they have a simple hydroxyl terminal group instead of an acetyl function. The OH is similar in weight and shape to a methyl function and thus is treated as the faster rotating terminal bond, i.e. the T_1 for C-1 is generated using the common D_i of the chain. Again, it is important to remember to correct the T_1 's of all CH and CH_3 carbons by multiplying by 1/2 and

3/2, respectively, in order to compare directly computer generated and experimental values. The respective anisotropic ratios for the cis and trans isomer are 50.5 and 79.3. The range over which the T_1 's were generated was 5-50 and 5-75, respectively. For both, the center of mass is located between C-6 and C-7 in the all-trans conformation. The T_1 's were generated in many small steps, with the range over which D_z , D_x and D_i were varied getting smaller and finer each run. The following results are a collation of all these runs and represents a large expense of computer time and cost.

The generation of the T_1 's was first carried out for the C-6 to C-1 (towards hydroxyl group) of the trans-tetradecen-1-ol, 7. Allowing for a 10% error in each of the six T_1 values and summing these, one would expect the best-fit parameter to be at least less than 1.52, or 0.25 per carbon, in order that the calculation be credible. Figure 4 shows the relationship between the best-fit parameter and the anisotropic ratio, and Figure 5 graphs the D_z , $D_x (=D_y)$ and D_i (leading to the best-fit parameters) against the anisotropic ratios. The large scatter in Figure 4, and the anisotropic ratios where the best-fit parameters are greater than the two adjacent points are indications that the diffusion coefficients have not been fully optimized. For an anisotropic ratio of less than 50, the best-fit parameter never goes to a true minimum. As an example

Figure 4. Best-fit parameter as a function of the anisotropic ratio for fitting C-6 to C-1 of 7.

Figure 5. The rotational diffusion coefficients as a function of anisotropic ratio for fitting C-6 to C-1 of 7.

of the generated values, compare them to the experimental values (1.98, 1.98, 1.98, 2.65, 3.14 and 3.42) at anisotropic ratios of 5, 17 and 32. At 5 the values are 1.77, 1.98, 2.39, 2.73, 3.08 and 3.43; at 17 they are 1.89, 2.00, 2.44, 2.75, 3.08 and 3.39; at 32 they are 1.89, 1.98, 2.47, 2.79, 3.13 and 3.44. Agreement is good at all anisotropic ratios and there is really only the value of C-4 which differs greatly. Since C-4 is part of a large multiplet it must be given the T_1 value measured for the multiplet when in fact, its value may be higher.

The T_1 's from C-7 towards the double bond of trans-11-tetradecen-1-ol were now calculated. An important physical property to note is the barrier to rotation of single bonds adjacent to a double bond. Due to the bond angles and carbon hybridization involved, the barrier to rotation is lowered to 2 kcal/mole while that of a single bond in an alkane chain is 3 kcal/mole(16). Thus, the rotation diffusion coefficients for the C-10 to C-11 and the C-12 to C-13 bonds, as well as that for the terminal C-13 to C-14 bond, should be larger than the common value for C-7 to C-10, and will have to be fitted individually. Molecular models confirm that there is a lessening of interactions. The C-11 to C-12 double bond will have a zero rotational diffusion coefficient.

The best-fit parameter versus the anisotropic ratio for the fitting of C-7 to C-10 inclusive is shown

as Figure 6. The plot of D_z , $D_x(=D_y)$ and D_i against the anisotropic ratio is shown on Figure 7. The values have not been fully optimized at every anisotropic ratio, but the agreement is very good. At an anisotropic ratio of 45 the generated T_1 's are 1.98, 1.97, 2.45 and 2.73, as compared to 1.98, 1.98, 2.52 and 2.71. If the same D_i is used for the C-10 to C-11 bond and zero for the D_i of C-11 to C-12, the predicted T_1 's of the C-11 and C-12 trans olefinic carbons are 3.25 and 3.15, respectively, as compared to the corrected experimental values of 3.60 and 3.24. The fact that C-11 and C-12 are too small could be corrected by entering a larger D_i for the C-10 to C-11 single bond. As predicted then, this bond rotates faster due to a decreased barrier to rotation. It is also interesting to note that the theory correctly predicts that the olefinic carbon further away from the center of mass will have a smaller T_1 than that nearer to it. No further work was carried out to fit the remaining two bonds in the chain.

The pattern for calculating the T_1 's of the cis-tetradecen-1-ol, 6, was the same except the range of the rotational diffusion coefficients was kept coarser and thus the best-fit parameters have not been completely optimized. First, the T_1 's for C-6 to C-1 (towards the hydroxyl end) were fitted. The plot of the best-fit

Figure 6. Best-fit parameter as a function of the anisotropic ratio for fitting C-7 to C-10 in 7.

Figure 7. The rotational diffusion coefficients as a function of anisotropic ratio for fitting, C-7 to C-10 in 7.

parameter against the anisotropic ratio is shown on Figure 8, and that of the corresponding D_z , $D_x (=D_y)$ and D_i versus anisotropic ratio on Figure 9. The minimum best-fit parameter for the six carbons of the cis isomer was a value of 0.92, as compared to 0.75 for the trans form.

When generating T_1 's towards the double bond of 6 the same strategy was employed as used in 7. The first four carbons, C-7 to C-10, were generated with one common D_i . The two graphs of the best-fit parameter and the rotational diffusion coefficients against the anisotropic ratio are included as Figure 10 and Figure 11, respectively. Less effort was put into minimizing the best-fit parameter but the trend is clear: the larger the anisotropic ratio, the worse the fit. Again one would expect that C-12 to C-13 and C-13 to C-14 would have to be fitted separately with a larger D_i than that for C-7 to C-10. The C-10 to C-11 bond should again have a 2 kcal/mole rotational barrier and thus it might be expected to have a larger D_i than the bulk CH_2 's. However, the corrected T_1 for C-11 is 2.84 (1/2 of 5.67) and it is lower than that of C-10. This suggested a greatly decreased rotation. On going to a molecular model it was observed that in this cis isomer rotation about the C-10 to C-11

Figure 8. Best-fit parameter as function of the anisotropic ratio for fitting C-6 to C-1 of 6.

Figure 9. The rotational diffusion coefficients as a function of anisotropic ratio for fitting C-6 to C-1 of 6.

Figure 10. Best-fit parameter as a function of the anisotropic ratio for fitting C-7 to C-10 of 6.

Figure.11. The rotational diffusional coefficients as a function of anisotropic ratio for fitting C-7 to C-10 of 6.

bond should not readily occur because of the resultant large interaction of C-13 and C-14 with the remainder of the molecule. If an individual D_i had been determined the results would have shown a very small D_i for this improbable rotation.

The results for the cis and trans isomers can now be compared. For the most part it is difficult to draw conclusions because the values are quite similar and there is a 10-15% error in the T_i values. The trans molecule is very much like a saturated alkane at both ends and this is reflected in the similarity of D_z , $D_x (=D_y)$ and D_i from each end. The cis molecule on the other hand will be like an alkane towards the hydroxyl end, but the spatial arrangement of the cis olefin results in many atoms lying off of the long axis and creating a "bulky" end. There should be greater resistance to D_i further up the chain in the C-7 to C-10 range since a much bulkier part would have to be rotated through space. The calculated D_i is significantly less here and it is the only case in either cis or trans where $D_i < D_x = D_y$ (see Figure 11). For the same cis molecule it is interesting to note that the D_z and $D_x (=D_y)$ predicted from the two ends are near identical. Other than the already noted D_i in the double bond end of the cis isomer, it is not possible to compare the cis and trans molecules further because of the similar values.

F. Summary

The preceding represents all the work that was performed in this project. As a summary, there are a few points which remain to be noted.

The results for the rotational diffusion coefficients of the three molecules studied qualitatively agree with the usual concept of the physical behavior of this type of molecule in solution. As the molecule becomes more linear, D_i must decrease since any internal motion will decrease the anisotropic ratio. With a decreased D_i the more linear molecule should prefer to rotate more rapidly about its long axis, i.e. increased D_z and decreased D_x , D_y . These trends can be noted in Figures 3, 5, 7, 9 and 11. The absolute values of the rotational diffusion coefficients are very similar to those obtained for the n-alkanes Levine examined(13). There have been other applications of this theory but in systems other than simple alkanes: for example, to proteins and protein sidechains, (17-23), to cycloalkanes (24,25), and to alkane derivatives (26,27).

During this current work a number of aspects have been identified for further consideration. These include:

- (i) For the molecules studied here, each end is treated separately as if it did not know

what happened beyond the center of mass.

However, the molecule moves as a whole and most likely has correlated motions.

- (ii) It has been assumed that the molecular axes of rotation will coincide with the principal molecular axes as determined from the moments of inertia. Or put another way, we assume the center of mass is stationary. This restriction will be more severe as the molecule distorts due to internal motions.
- (iii) There is no way of indicating the difference between a cis and a trans double bond. The angles entered are complimentary and thus the same T_1 's are predicted for both conformations.
- (iv) The plot of D_z , $D_x (=D_y)$ and D_i against the anisotropic ratio would still be reasonable after exceeding the maximum anisotropic ratio calculated for each molecule. Is there any reason for limiting the ratio to that predicted by the moments of inertia?
- (v) Although there is a solution for fitting the T_1 's over a wide range of anisotropic ratios, is there a physical significance to the minimum

observed for the plots of best-fit parameter against the anisotropic ratio? Since internal motions can occur, the all-trans conformation can not be a realistic picture of the molecule in solution. It is possible that the minimum reflects the shape the molecule takes on a time average.

- (vi) No consideration has been given to any form of solvent-solute interaction. Any hydrogen bonding or physical interactions will alter all parameters.

All of the above questions and the previously identified assumptions must be considered when applying this analysis to the T_1 's of any molecule. Also, it is important to remember that the above calculations require considerable computing time and money and researcher time (when using the program in an interactive mode at a computer terminal). Of significance is the conclusion of Deslauriers and Somorjai (18,19) from their studies of peptides. They concluded that it would not be possible to calculate the conformation of a molecule from its T_1 's, and that this would be especially true

for small molecules. There have been other modifications suggested (28-30) which would remove some of the assumptions and restrictions invoked by Levine but these would undoubtedly increase the time, cost and effort needed to analyze molecules such as the pheromones. It will be important to choose carefully the systems to which such analyses are applied.

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