Recently a Ph.D from our lab (Shikha) has been awarded for the work on progesterone; the present dissertation on the studies with testosterone is in fact parallel to that work. Therefore, review for some work may be common for both. The target molecule testosterone is expected to encompass the spectroscopic features of simple keto chromophore e.g., >C=O and these transitions are in turn significantly modified in various ways if this keto group is in conjugation e.g., mesityl oxide. This conjugation affects physical, chemical, structural and spectroscopic properties depending upon the extent of conjugation. We have focused mainly on the structural properties and corresponding spectroscopic properties. The conjugation may result in setting up new equilibria e.g., between conjugated and unconjugated species, keto-enol tautomerism (if facile protons are in the immediate neighbourhood) and the possible formation of charge transfer species. The number of transitions will certainly increase related to all possible new species. The spectroscopic part which is of our interest will show; (i) red shift in the main bands, (ii) the change in intensity and (iii) the presence of new bands. If this keto group and conjugation are part of cyclic structure e.g., cyclohexenone, the transitions are further modified. The additional substitution particularly condensation of additional rings e.g., steroids (testosterone) will affect transitions as per Woodwards Hoffman rule. If this additional substitution have H atoms or methyl groups in the plane or perpendicular to the axis may cause isomerisation. If additional fused rings are twisted at some points further isomerisation may results. All such changes will complicate transitions and it becomes a challenge to sort it out. These changes introduce some time minor effects or subtle changes, which no tool of analytical chemistry can sort out. The usual practice in chemistry particularly with bigger and substituted molecule is to pay more attention to the head of the molecule and in the majority of the cases the tail is either neglected or scant attention is paid to the tail. Though, this is the only difference which separates testosterone and progesterone, but introduces great difference in their physiology.
Therefore, we felt that both ends of the molecule should be looked into and only then the conclusions drawn appear logical. Our target molecule testosterone has substitution of OH group at C17, our broader thinking to treat this molecule as substituted cyclohexenone doesn’t seem unreasonable. Before, we present the summary of relevant works it would be desirable to proceed along the following lines;

2.1. Derivative Spectroscopy

The major thrust of the present project is the exploration of the various dimensions of this powerful technique to reveal and unearth the various spectroscopic features associated as an integral part of keto chromophore.

The basic features of this technique have already been incorporated in Introduction chapter.

Till 1985, the derivatisation of simple spectral curve was limited because of some serious limitations in the instrumentations, e.g., gratings with low resolving power could not resolve closely spaced bands, the data recording was mainly in analogue mode which needed devices to digitize data, limited optical sophistication, less sensitive electronic circuitry, limited power of computers for data processing, storage and transformation of data from one form to other. The biggest challenge of all was the limited array of softwares with limited data transformation and processing facilities. Even then this technique had extremely limited applicability because higher order spectra were not possible and the level of noise raised serious questions about the real benefit of this technique. Hence, the level of skepticism was high. Anthony J. Owen from Varian Associates first published a note and demonstrated the versatility of this technique [1-3] and published the second derivative spectrum of testosterone (without any explanation) and demonstrated a set of bands in the long wavelength region of the normal zero derivative spectrum which had only a very broad peak with some shoulders. This small article worked like a catalyst and inspired us to make extensive efforts to make this technique workable, particularly with higher order of derivatives. The choice of steroids was a kind of personal fascination. Sanchez et al has really done a remarkable job in
reviewing all the work reported using derivative spectroscopy. They published series of review articles. They reviewed the work till 1988 [4]. It summarized some related theoretical aspects, some of the instrumental devices and mainly analytical application of this technique. The next reviewed the work till 1995 [5], the third one till 2004 and the last one till 2010 [6, 7]. Antonov et al [8] have laid a sound mathematical background to explain the pros and cons of this technique. Now, this technique has blown up to its full potential and is being used in various fields. From all these publications we have noticed certain commonalities; (i) majority of workers have confined to either first or second order derivatives with very few using third derivative spectra, (ii) almost all the workers have used its quantitative application; determining the components in the mixtures with remarkable accuracy. Almost all of them have determined the amplitude of signal by measuring the height of the derivative signals from maxima to minima e.g., V. Pucci et al used the values from the derivative spectra for quantitative determinations as the difference in between the height of maximum at $\lambda_{\text{max}} = 227.2$ nm and that of minimum at $\lambda_{\text{max}} = 253.6$ nm of the first derivative spectra of progesterone.

This height is directly proportional to the concentration of the analyte and in literature mathematical justification has been given [9].

We managed to unearth quite significant number of papers in which researchers have employed derivative technique, but the kind of studies they were involved are not very relevant to the kind of studies we were involved in; hence we are skipping the reviewing of their work.

In between Karpinska published another review [10]. Besides reviewing the work of others, he cautioned researchers using derivative technique to be extra careful at least on some accounts; (i) same instrument and same software should be used for whole work, because each software may be performing similar task but the architecture, steps and most importantly the mathematical procedure and sequences may vary slightly. Even this small variation may have significant impact particularly in higher derivative spectra, (ii) for finer comparison between spectra, same set of parameters should be used for recording both spectra, (iii) all efforts should be made to reduce the noise to its lowest
level while recording the zero derivative spectra because sharp noise signals produces more intense bands on derivatization and in some cases where the real bands (in zero derivative mode) are weaker and broader are completely eliminated in higher order derivative as a matrix background noise. In this way one may miss real signals. He and others agree that careful smoothening of the bands before derivatisation eliminates some features in the beginning and at the end of spectra but the position of the bands in the central part of the spectra either do not shift or if there is any shift it is insignificant (provided noise level is very low). Therefore, we were extra cautious and made all out efforts to reduce noise to the lowest possible limit (not at the cost of eliminating real or important signals) and our results are highly reproducible. We have used with fair amount of success another criterion. We made to overlap the II\textsuperscript{nd} and IV\textsuperscript{th} derivative spectra smoothened using identical parameters. II\textsuperscript{nd} and IV\textsuperscript{th} derivative spectra of real bands bear a mirror image relationship while noise signals do not.

To the best of our knowledge not many researchers have used this technique to study, (i) Protonation and deprotonation studies in the presence of acids and bases, (ii) study of spectroscopic distinction between isomers e.g., keto-enol tautomerism, (iii) the presence of other molecules which are part of the chemical equilibria with the main molecules, and (iv) spectroscopic study and identification of different epimers in equilibria with parent molecule, and (v) the presence of vibrational bands associated with the electronic transition. We have made extensive efforts to explore these properties with the target molecule and with other molecules having some spectroscopic commonalities with the target molecule. Hence our study assumes significance.

Olson et al [11] have demonstrated the effects of structural variation through the application of derivative spectroscopy with limited success in the $n\rightarrow\pi^*$ transition of some keto-steroids and stated that it is possible to distinguish between $\alpha$-$\beta$-epimers (if structural variation is significant) as well as to locate the position of substitution in some cases. He also observed shifts of bands towards shorter wavelengths and marked decrease in the fine structure (without any suitable and satisfactory explanation) of the spectrum in polar
solvents. Major substitution at the C17 position in the steroid nucleus affected the electronic transitions of the 3-keto-\( \Delta^4 \) system in the ring A.

Struck et al [12] reported the fairly exact wavelength correlation between the first derivative of an UV spectrum and the fine structure of an optical rotatory dispersion curve, though both techniques have different principle of operation and functioning. Thus both techniques supplement each other as structural tools. In some cases lot of information have been gained about the structure of the steroid from its ultraviolet derivative spectrum in the 1st derivative mode and from its rotatory dispersion curve [11].

2.2. Difference Spectroscopy

John S. Vrettos et al [13] have shown that difference spectroscopy can be useful in highlighting and extracting small changes observed in spectra. The method can be applied to the analysis of any set of spectra for which an appropriate reference state spectrum can be obtained. Difference spectra requires no empirical input parameters to be computed do not amplify noise or sharp peaks and can be qualtitated for comparison with other spectra or analytical data. Difference spectra are a useful complement to other methods for analyzing spectroscopic data.

2.3. Solvent Effect

Out of the many properties of the solvents that affect dramatically the position of the bands, two properties e.g., dielectric behavior (polarization forces) and hydrogen bonding capability have been thoroughly studied and voluminous data is available in literature. Among all the chromophores, the keto group has attracted the attention of large number of workers [14, 15, 16]. We are including some classic reviews.

Burawoy [17], et al were the earlier workers to carry out detailed and systematic attempts to classify the effect of the solvent on the ultraviolet absorption spectra. They noted the shifts of absorption bands of different regions with the change of solvent polarity. A rather general and comprehensive treatment of solvent effect was presented by Bayliss and Mc Rae [18] who took into consideration the role of Frank Condon
principle and indicated that when an excitation occurs the solvation arrangement of both
an excited and ground state of solute molecule must be taken into account. McRae put the
theory on Quantitative basis and derived an expression to include dispersion forces,
permanent and induced dipoles in the solute and solvent molecules.

Kosower [19] reported a well defined pattern of spectral changes with changes in
solvent polarity for molecules or ion-pairs which are strongly polar in the ground state,
but far less so in the excited state.

Planar conjugated organic molecules containing double or triple bonded hetero
atoms (=O, =S, ≡N) frequently show singlet-singlet \( n \rightarrow \pi^* \) transitions [20].

Solvation effects can be usefully employed for distinguishing between \( n \rightarrow \pi^* \) and
\( \pi \rightarrow \pi^* \) transitions. Kasha [21] formulated a semi empirical rule that “With the increase in
the polarity of the solvents \( n \rightarrow \pi^* \) transitions show blue shift and \( \pi \rightarrow \pi^* \) transitions show
red shift”.

In the ground state for polar solute molecules, the solvent molecules orient
themselves around the solute molecules so as to bind most strongly with the ground state
charge distribution. If the excited state charge distribution of the solute molecule is
markedly different from the ground state charge distribution, then by Frank Condon
principal it is possible that the polar solvent molecules will not have the proper positions
and orientations to bind most strongly with excited state charge distribution. This condition
would give rise to a blue shift band since the solvation energy of the solute in excited state
may be less than the solvation energy in the ground state (relative to inert or non polar
solvents). Platt [20] similarly advanced an argument on the basis of relative charge
distribution, “\( n \rightarrow \pi^* \) transitions are weak and symmetry forbidden because the ground and
excited state charge distribution are markedly different. For \( n \rightarrow \pi^* \) transitions the excited
state charge distribution has a node through the molecular plane whereas the ground state
charge distribution has no node. Therefore “electronic transitions in molecules which have
low intensities because the ground and excited state charge distributions do not overlap
strongly are likely to give blue shift bands.” [22]
The $\pi\rightarrow\pi^*$ transitions for many compounds e.g., ketones in hydroxylic solvents, the H-bond persists in both the ground and excited state and relatively the greater stabilization of more polar excited state as compared to the ground state leads to the red shift. This behavior persists in polar solvents also. The reverse of this phenomenon occurs in $n\rightarrow\pi^*$ transitions [23].

2.3.1 Solute–Solute Dimer

T.F. Lin et al reported the solute-solute type dimerization of acetone in hexadecane and CCl₄. Their finding is based on the change in the bond lengths of the keto group and enthalpy change of the order of -2.8 kcal/mol as shown below:

![Figure 2.1: Acetone Dimer](image)

The actual geometric structure is probably not a rigid anti-parallel arrangement of two dipoles and undoubtedly many variations from the anti-parallel alignment are possible [24, 25]. They were of the opinion that perhaps the solute-solute dimerization may be a general property of carbonyl compounds. In fact [26] the solute-solute dimerization of testosterone in the solid state has been reported.

2.3.2 Vibronic Coupling

Eastwood et al [27] studied the spectra of acetone in the vapor phase and showed the probability of the carbonyl valence vibrations in the excited state with approximate values 280, 272, 264 nm with frequency separations of 1107 cm⁻¹. This was really a
pioneering work which laid the foundation to locate and identify vibrational bands associated with electronic transition for keto groups.

Henderson et al [28] postulated that the selective absorptions in ketones are caused by intramolecular vibrations taking place primarily within the carbonyl group (oscillation centre) due to alternate formation and breaking down of unstable ring system. The momentary formation of ring is affected by free partial valences which show their appearance under specific conditions due to which electronic disturbance occur between two phases accompanying the oscillation causing selective absorptions in ketones e.g. like in acetone.

![Tautomerism in Ketones](image)

**Figure 2.2**: Tautomerism in Ketones

2.4. α,β-Unsaturated Enones

The absorption spectra of the α,β-unsaturated carbonyl compounds generally show only one broad band. However the lack of symmetry of this band in many spectra suggests that more than one transition may be involved. In some cases presence of small shoulder on the short wavelength side of the broad band has been observed. The comparison of ORD and CD curves of α,β-unsaturated ketones and structurally related dienes indicates the presence of two close lying transitions in the former compounds [29].

VESCI-CI calculations strongly suggests that the broad UV absorption bands observed for α,β-unsaturated ketones is generally made up of two \( n \rightarrow n^* \) transitions[30]. There are in general two \( n \rightarrow n^* \) transitions; one in the 200-220 nm range and other in the
220-260 nm range for $\alpha,\beta$-unsaturated carbonyl compounds which are usually comparable in intensity and too close to be resolved and discussed in terms of intensity change only [31].

The ultraviolet spectra of conjugated isomer in iso-octane exhibits bands at 231 nm and 329 nm (extinction coefficient $10 \times 10^4$ and 40.5 liters/mole cm$^{-1}$) respectively.

Mesityl oxide (conjugated form) has two isomeric forms; syn-anti with relative stability dependant on the solvent. Isomer syn is more stable by a $\Delta G$ (syn-anti) = 2.4 kcal/mol. The best result for the isolated MO was $43.5 \times 10^3$ cm$^{-1}$ (229.8 nm) obtained with TD/B3LYP/aug-cc-pVTZ for the syn isomer which is in good agreement with experimental results measured in iso-octane solution. MO modifies its conformation from syn to anti when changing to a more polar solvent [32].

The intensities of IR and UV absorptions have been especially useful for distinguishing between s-cis and s-trans conformers [30]. Liljefors and Allinger [33] suggested that in $\alpha,\beta$-unsaturated ketones, s-cis conformation absorbs at longer wavelength than the s-trans conformation, the difference in energy being 0.3-0.4 eV (corresponding to 10-20 nm in the wavelength interval 200-250 nm, where most of these compounds have absorption maxima in ethanol). Bienvenue [34] in an attempt to correlate observed transition energies with relative concentrations as obtained from IR measurements arrived at the s-cis/s-trans absorption energy difference as 2400 cm$^{-1}$ (0.30eV) in good agreement with calculations.

In mesityl oxide the wave length of absorption for syn and anti conformer in terms of wavelength may be small or large depending upon torsional angle. Therefore the extinction coefficient varies with this torsional angle and the separation between the bands also varies. One may see the single band which is unresolved or two separate bands [33].

According to S. Nagakura, et al [35] when acetone is taken in aqueous sulphuric acid, with the increase of acid concentration a new band appears at 231 nm may be the
band of mesityl oxide, and which shows a large red shift in sulfuric acid. From the resonance viewpoint, this absorption seems to correspond to the transition between two levels caused by the resonance between the nonpolar structure and the polar structure as shown below:

![Equation 1](image)

**Equation 1**

An isosbestic point would most probably mean the co-existence of only two kinds of solute species in the solution. Similar isosbestic point is observed for mesityl oxide and acetophenone with the ethanol concentration from 0 to 80%. In this case one of the species is free ketone and the other is H-Bonded ketone. These two kinds of species will show two different kind of $n \rightarrow \pi^*$ spectra [36].

Deno reported that $\alpha, \beta$-unsaturated ketones in concentrated sulfuric acid solutions exist as mesomeric carbonium ions [37]:

![Equation 2](image)

**Equation 2**

2.4.1. Cyclohexenone (Cyclic $\alpha, \beta$-Unsaturated Ketones)

The review of this molecule is most important for us because with this molecule we have laid the foundation.

Kosower et al [38] reported the UV spectra of cyclohexenone in various solvents. Two transitions; highly intense $\pi \rightarrow \pi^*$ at 217.2 nm in isooctane, $\varepsilon_{\text{max}} = \text{ca. 12,000}$; (230 nm in water) and very weak $n \rightarrow \pi^*$ at 342 nm in isooctane, $\varepsilon_{\text{max}} = \text{ca 27}$; (307 nm in water) were
observed. The $\lambda_{\text{max}}$ for both these transitions lies in between for solvents with intermediate polarity. It is clear that $\pi\rightarrow\pi^*$ shows red shift and $n\rightarrow\pi^*$ shows blue shift with the increase in the polarity of the solvents; a characteristic feature of such transitions.

Allinger et al [39] observed broad UV absorption band in number of $\alpha,\beta$-unsaturated ketones and reported that it is actually due to two close lying $\pi\rightarrow\pi^*$ transitions as observed by ORD and CD. According to them out of two one transition is strongly allowed for near planar systems and second one is weakly allowed, this allowedness relationship interchanges as the dihedral angles between the chromophores goes to 90° [33]. The inspection of Eigen vectors of the two states shows the major contribution arises from the highest filled to the lowest unfilled orbital in both the cases [30]. H. Ziffer and C.H. Robinson [40] determined the CD curves of numbers of $\alpha,\beta$-unsaturated ketones which revealed the presence of new very strong optically active transition near to that of $\pi\rightarrow\pi^*$ transition in the range of 200-220 nm. the band at ~215 nm indicates that its rotational strength is comparable to that of $\pi\rightarrow\pi^*$ transition. However UV absorption spectra indicate that the transition dipole strength is very weak.

On comparing the cyclic and acyclic $\alpha,\beta$-unsaturated ketones, longest wavelength $n\rightarrow\pi^*$ transitions, it was found that the effect of conformational change twisted S-cis→planar S-trans which is about 6 nm, is compensated by ring closure effect [33].

### 2.4.1.1 Study of Cyclohexenone by CD Spectroscopy

An empirical analysis of CD spectra of variety of cyclohexenone, including variable temperature and solvent studies reveals the presence of up to three cotton effects in the 260-185nm region, in addition to the well recognized $n\rightarrow\pi^*$ cotton effect at 300-350 nm. The maximum of the broad $n\rightarrow\pi^*$ band appears in UV and CD spectra between 370-290 nm being blue shifted in solvent of high polarity. At shorter wavelength 185-260nm: Band I appears in both in UV and CD between 220-260 nm which undoubtedly belongs to allowed $n\rightarrow\pi^*$ transitions (K band).

Later on Gawronski [41] tried to isolate two bands through application of CD technique. For cyclohexenone he showed three bands in both CD and UV; band I-220-
260 nm (belongs to allowed $\pi \rightarrow \pi^*$ transition) band II-200-220 nm (formally weakly allowed $\pi \rightarrow \pi^*$ transition), usually both bands are better separated in non polar solvent. Band I and Band II shows the bathochromic shift with solvent polarity, indicating the large bathochromic shift in Band II maxima with change in solvent polarity.

The long wavelength bands I and II are formally ascribed respectively first allowed and second formally forbidden $n \rightarrow n^*$ transitions. The third band III cotton effect below 200 nm is attributed to $n \rightarrow \sigma^*$ transition in the CO group. If this is the case then we too should expect such kind of behavior with our target molecule also.

Band II appearing in CD spectra in the range 200-220 nm remained highly speculative until recently Allinger and Liljefor published the result of the VESCF-CI calculations on conformations and electronic absorption spectra of $\alpha,\beta$-unsaturated ketones and aldehydes [30,42].

In case of cyclic enones for e.g. cyclohexenone, the particular band position I and II are strictly related to the substitution pattern. In planer or nearly planer enones the II$^{\text{nd}}$ $n \rightarrow n^*$ transitions is forbidden and does not contribute to the experimental UV results in the absence of non bonded interactions. The most stable conformations of the cyclohexenone ring in this class are half chair or sofa. The preferred cyclohexenone conformation appears to be reflected by the band I cotton effect. In many cases where the available X ray data suggest planar sofa conformation, the band I cotton effect is weak, often seen as a shoulder on the strong band II cotton effect of the same sign [43].

2.4.1.2 Hydrogen Bonding in Cyclohexenone

Kosower [38] studied the UV spectra of cyclohexenone in number of solvents; hydroxylic and non-hydroxylic. The observed red shift in $\pi \rightarrow \pi^*$ have been related with the Z-factor; a polarity based constant devised by Kosower himself. He observed linear relationship between the band position and Z- value. As the Z-value increases from non-polar isoocutane ($Z = 60.1$) to water highly polar ($Z = 94.3$), the band progressively shifts to longer wavelength and reverse of this behavior was observed in the case of $n \rightarrow \pi^*$
transitions. Surprisingly, he didn’t mention the role of hydrogen bonding with hydroxylic solvents. It appears that he was of the opinion that band positions shifts in such compounds is dominated by polarity considerations only and hydrogen bonding does not make any contribution. Though, it is very well accepted and known in UV spectroscopy that hydrogen bonding with hydroxylic solvents plays a very significant role. Hydrogen bonding and higher dielectric constant both play their role in bands positions. It appears that in such compounds hydrogen bonding is not playing any role and it is only the higher polarity (higher Z-value) that fixes the band positions in the spectrum. we feel that, In all likelihood conjugation causes charge separation and if this is the case then polarity interaction will strongly affect both excited as well as ground state to a larger extent as compared to hydrogen bonding and this view is supported by Jaffe as well.

Dimerization

The biological activity of macromolecules is known to be dependent on hydrogen bonding [44]. It is well known that hormones play very vital role in many biological activities, It appears that the studies of dimerization (solute-solute) with progesterone through normal techniques e.g., UV, NMR etc. didn’t yield any worthwhile information, while for testosterone a weak dimerization is reported and accepted. Ribeiro-Claro et al [45] managed some interesting results through IR. They took up cyclohexenone molecule, because it is the most important moiety of most of the hormones.

![Chemical Structure of Cyclohexenone](image)

**Figure 2.3:** Chemical Structure of Cyclohexenone
They observed dimerization involving C-H⋯O type of hydrogen bonds in liquid phase through two sites in the molecule; C₂ site involving C\text{sp}²–H bonding and C\text{sp}³–H type of bonding at C₆. The wave number shifts upon hydrogen bonding have been evaluated. The carbonyl νC=O stretching mode is predicted to be red-shifted (from dimer to monomer) by up to about -20 cm⁻¹ depending on the dimer form. The effect on the νC–H modes is less well defined: the modes involving the C\text{sp}³–H donors are generally blue-shifted by about 6–9 cm⁻¹; while those involving C\text{sp}²–H donors present low dimerization shifts, in the -4 cm⁻¹ to +2 cm⁻¹ range. Their findings are well supported by thermodynamic calculations. The presence of a pseudo-isosbestic point in the νC=O region supports the assignment of the two observed bands to two species in equilibrium, considered to be the free and 1:1 associated forms.

2.4.2. Testosterone (α,β-unsaturated Keto Steroid)

Kosower [16] extended this study to unsubstituted bicyclic α,β-unsaturated ketones i.e. bicycle [4,4,0] dec-6-en-8-one. The presence of keto group and conjugation on one moiety actually makes A ring and the other simple six membered moiety is B ring in our target molecule steronone. Therefore, their study is quite relevant to our postulations. For this bicyclic compound they have reported the λ\text{max} for π→π* transition in cyclohexane at 229 nm (at 245.5 nm in water, ε\text{max} = 16500) and λ\text{max} for n→π* transition at 337 nm (ε\text{max} = 37) in cyclohexane (ca. 304 nm in water). On comparison it is clear that in bicyclic compound both bands have definitely red shifted. They have postulated that with substitution the electrostatic repulsion should destabilize both the ground and excited state and this should result in the shifting of both these bands (this destabilization is strong if the substituent is a heteroatom). The π→π* transition of the, β-unsaturated ketones leads to an excited state with the greater contribution from the dipolar resonance form, C⁺−C=C−O− than present in the ground state.

They calculated the dielectric constant for the ground and excited states as 4.0D and 9.6D a significant change in the transition dipole moments; a sign of intense transition. They have further postulated the redistribution of π-electron density in
conjugated positions and calculated a charge distribution of 0.24 in the ground state and 0.56 in the excited state, with a possible resonance structure as shown below:

![Equation 3](attachment:image.png)

If this ionic structure also persists in solution phase even momentarily then this structure too should show either its own band or a shift in the band positions. If we apply Woodward’s and Fieser rule on cyclohexenone to calculate the band positions for simple bicyclic ketones then red shift of the order of ~ 15 nm for bicyclic compounds should be observed and they have observed it. It can be inferred that further substitution should not shift band positions as they will be too far from chromophore. If this logic is extended and applied on to our target molecule testosterone, the band positions for both $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transitions should not be shifted (as compared to bicyclic ketone). Indeed this we have observed.

Louis Dorfman reviewed the UV spectroscopic studies of steroids in a classic paper [46]. He reported the first such thorough study of UV spectra of $\alpha,\beta$-unsaturated ketones particularly testosterone carried out by Woodward et al [47]. Woodward calculated the UV spectra of testosterone at $\lambda_{\text{max}}$ 244 nm and experimentally the bands were observed at $\lambda_{\text{max}}$ 241 nm and $\lambda_{\text{max}}$ 306 nm with extinction co-efficient of 16,600 M$^{-1}$cm$^{-1}$ and 94 M$^{-1}$ cm$^{-1}$ respectively for $\pi-\pi^*$ and n-$\pi^*$ transitions. Woodward in satisfactory manners postulated the appearance of bands at these wavelengths. He also studied the solvent effect on the position of UV bands. In general $\alpha,\beta$-unsaturated keto steroids resemble closely to simple $\alpha,\beta$-unsaturated carbonyl compounds [48].

The number of steroids containing the $\alpha,\beta$-unsaturated keto structure can be treated as an extension of the 2-cyclohexen-1-one system suggesting that the relationship is probably valid [49]. A chemical approach to the problem of testosterone assay was
made by Reynolds and Ginsburg [50] who devoted a quantitative method based on ultraviolet absorption at 240nm of Δ^4-3-ketosteroids like testosterone.

Haskin (1950) considered that the relatively high extinction coefficient at 240 nm made testosterone readily measurable at optimum concentrations of 1-20μg/ml of ethanol. [51]. The spectrophotometric assays, using the direct UV spectra, were performed by V. Pucci et al (2003) at the wavelength of 240 nm. The values of derivative used for quantitative determinations were calculated as the difference between the height of the maximum at $\lambda_{\text{max}} = 253.6$ nm and that of the minimum at $\lambda_{\text{max}} = 227.2$ nm of the first derivative spectra. 2-propanol was used as the blank solvent for all measurements [52].

### 2.4.2.1 Quantum Mechanical Studies

Geometries and electronic structure of Progesterone, testosterone and some related steroids were assessed by ab initio calculations using the 6-31G* basis set [53]. For these two compounds it was shown that both HOMO and LUMO are located at the A ring, the pi bond at C₄, C₅, and the carbonyl at C₃.

Experimentally [54], it has been demonstrated that progesterone has slightly higher electron affinity than many of its structural analogues e.g, testosterone. The high electronic density and frontier orbitals of progesterone located at C₄-C₅ show electron delocalization along the (O=C₃-C₄=C₅) steroids yielding 5α- and 5β-reduced metabolites.

\[
5\beta\text{-reduced} \rightarrow \text{Cis configuration} \rightarrow \text{less stable} \rightarrow \text{highly reactive}
\]

\[
5\alpha\text{-reduced} \rightarrow \text{trans configuration} \rightarrow \text{Stable} \rightarrow \text{Less reactive}
\]

### 2.4.2.2 H-Bonding

H-bonding studies at various levels with different objective were carried out. Weak chain cluster was observed in the CSI mass spectra of testosterone; this result is consistent with the X-Ray analysis findings [55]. This basically means that dimerization of testosterone is through H-bonding.
Since progesterone bears two potential hydrogen bond acceptor groups corresponding to the oxygen of C\textsubscript{3}=O and OH at C\textsubscript{17}, H-bond basicity appears again to be an important property of testosterone.

From ab initio calculations it is found that electrostatic potential is more negative around conjugated carbonyl i.e. C\textsubscript{3}=O, the difference of about 7 kcal mol\textsuperscript{-1} shows that electrostatic component of hydrogen bonding will favor the fixation of H-bond donor to O\textsubscript{3}. From electro-potential iso-surface results, it was found that the lone pair density is seen to be continuous around O\textsubscript{3}, therefore more space is available for approaching the H-bond donors around O\textsubscript{3}.

2.4.2.3 Protonation Studies

Zalewski and Dunn [48] have reported the protonation of \(\alpha,\beta\)-unsaturated alicyclic ketones and \(\alpha,\beta\)-unsaturated ketosteroids in sulphuric acid solutions [50]. They observed that \(\pi-\pi^*\) band shifts bathochromically with increasing solvent polarity and further bathochromic shift occurs in the sulphuric acid solutions. The shift continues in increasing concentrations of sulfuric acid, so that the absorption curves do not pass through a single isosbestic point as the carbonyl compound B is protonated to BH\textsuperscript{+}.

The \(\lambda_{\text{max}}\) and \(\varepsilon_{\text{max}}\) of progesterone in 72\% perchloric acid shifts in the beginning from 240 nm to 292 nm \((\varepsilon = 4.3 \times 10^2)\) and then it is practically constant even after 60 and 120 min while the absorptivity of testosterone and its derivatives changes continuously with time. The position of \(\lambda_{\text{max}}\) of progesterone in perchloric acid is same as it is observed in conc. sulphuric acid at zero time [56]. Dilution with water causes slight hypsochromic and hypochromic shift [57].

The absorption maximum of the steroid in 10 M HCl was shifted to 258 nm, and a shoulder at 285 nm. When the steroid was added to a stronger acid, namely, 10 M H\textsubscript{2}SO\textsubscript{4}, the peak at 258 nm decreased, becoming a shoulder on a larger peak which absorbed maximally at 285 nm. The spectral changes in acid were stable for at least 24 h at all concentrations of acid and could be completely abolished by neutralization with equivalent amounts of NaOH. This suggests that changes are reversible i.e., only simple
protonation. The absorption spectrum of 19-nortestosterone in acid suggests the sequential formation of two species, one of which is a precursor of the other. The first species ($\lambda_{\text{max}} = 258$ nm) is likely to be the protonated carbonyl form of the steroid, whereas the second is a more highly conjugated species, such as a dienol. Since the latter species absorbs at 285 nm and has almost the same extinction coefficient as the parent compound, it is likely to be a homoannular dienol with double bonds at 2(3) and 4(5) rather than a heteroannular structure with double bonds at 3(4) and 5(6), since the latter would be expected to absorb at lower wavelengths and have a higher extinction coefficient than the parent compound (Fieser & Fieser, 1959; Dorfman, 1953) [57].

![Equation 4](image)

### 2.4.2.4 CD Spectroscopic Study of $\alpha,\beta$-Unsaturated Steroids

The recent linear and CD studies of the number of steroidal $\alpha,\beta$-unsaturated ketones confirmed the presence of an additional $\pi\rightarrow\pi^*$ transitions around 200 nm, correlated well with calculations including all singly and doubly excited configurations [58].

Beecham et al [59] recorded the CD spectra of 22 steroidal 4-en-3-ones in cyclohexane solution through the wavelength range 400-265 nm and showed that both the singlet-triplet and the singlet-singlet $\pi\rightarrow\pi^*$ electronic transitions contribute. In the case of Androst-4-en-3-one and its 17$^{\text{th}}$ substituted derivatives there is characteristic CD spectrum through the $\pi\rightarrow\pi^*$ region. In hydrocarbon solutions these compounds show negative cotton effect through the range 380-265 nm, the band envelop in this region is
highly structured showing 7-8 identifiable bands assigned to a vibrational progression in the carbonyl stretching mode of the upper state with the intervals of 1200 cm\(^{-1}\). The cotton effect maximum being the third lowest in energy at 339 nm with \(\Delta\varepsilon\cdot c\) -1.2 to -1.4. Many of the spectra also exhibit a positive band at 384 nm \(\Delta\varepsilon\cdot c\) +0.01 this feature was first remarked on by Kuball, who observed it in spectra from testosterone acetate and propionate both in n heptane. Beecham and Collins believed it to be the part of \(n \rightarrow \pi^*\) transition, with different origin namely singlet -triplet \(n \rightarrow \pi^*\) transition.

This has also been identified by Kearns in the UV absorption spectra of steroidal 4-en-3-ones [60]. The cd intensity of the positive band at 384 nm is two to three orders of magnitude lower than that of the negative band at 339 nm. In the absorption spectra of carbonyl compound this is the sort of intensity difference to be expected between the singlet -singlet \(n \rightarrow \pi^*\) and the singlet -triplet \(n \rightarrow \pi^*\) transition and the latter being strictly forbidden (spin forbidden) and if observable at all it is only through intensity borrowing [59].

Beecham and Hurley [61] studied the hydrogen bonding and the \(n \rightarrow \pi^*\) blue shift in \(\alpha,\beta\)-unsaturated ketones and reported that the frequency shift due to H-bonding is small and that the apparently large H-bonding contribution to the blue shift results mainly from a redistribution of intensity between vibrational sub bands.

In the aliphatic hydrocarbon solvents, the vibrational structure of the \(n \rightarrow \pi^*\) electronic transitions bands in the CD spectra of \(\alpha,\beta\)-unsaturated ketones is remarkably well defined; same is the case for steroidal enones. In the mixture of ethanol and cyclohexane solvents at different volume percentage the amplitude of vibrational sub bands changes disproportionately with changing ethanol content and shows an isosbestic point which suggests the presence of only two interconvertible, circularly dichoric solute species at ethanol concentrations up to 2%. They have identified the vibrational shift of the order of 1200 cm\(^{-1}\) as due to free (non hydrogen bonded) specie in the ground state and remarked further that the vibrational splitting with the hydrogen bonded specie in the excited state is also similar; it is only the intensity that changes.
The curves of both the free and 100% H-bonded solute encompass the same spectral region (394-282 nm) and both exhibit 8 evenly spaced vibrational sub bands. For the free solute the 1200 cm$^{-1}$ sub band spacing has been identified as the frequency in the excited state of the carbonyl stretching mode. The H-bonded solute exhibit the same vibrational progression, only slightly modified in overall intensity, in energy and in energy difference between vibrational levels. The progression is however, extensively modified in the distribution of between the individual bands. One result of redistribution of energy is that the electronic band maximum moves from the third vibrational peak to the fourth, so that almost all the apparent energy difference between the two spectra is simply the energy difference between adjacent vibrational peaks.

Antonio Sanchez Bueno et al [62] has studied conformation of testosterone molecule with the help of CD spectroscopy in the range 260-360 nm, where he reported the positive cotton effect at 275 nm due to n$\rightarrow$$\pi^*$ transitions of saturated ketones and a negative one at 315 nm due to $\pi$$\rightarrow$$\pi^*$ transition of the $\alpha$$,\beta$-unsaturated keto group of testosterone. And the above results were well supported by previous works [63], Gawronski and Brunke suggested that the remote substituents in the polycyclic systems have little influence on the chiroptical properties of enone chromophore. There is essentially no difference between the CD spectra of the steroidal 4-en-3-ones with or without side chain at C$_{17}$ [64,65].
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