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Proton Magnetic Resonance Study of Angiotensin II (Asn^1Val^5) in Aqueous Solution†

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ABSTRACT: All the resolved resonances in the 220-MHz proton magnetic resonance (pmr) spectrum of angiotensin II (Asn^1Val^5) (AII′) in D_2O have been assigned to specific hydrogens. In H_2O additional assignments were made of resonances originating from peptide NH hydrogens of Phe and Arg, primary amide NH hydrogens of Asn, and the four equivalent guanidino NH hydrogens of Arg. Conformational transitions associated with titration of the α-amino and/or the imidazole group(s) ($pK_a = 6.6 \pm 0.2$) and with titration of the phenol group ($pK_a = 10.2 \pm 0.2$) have been confirmed. Pmr determined pK_a values in H_2O at 23° and in D_2O at 4° (shown in parentheses) are all normal: carboxyl 3.07 ± 0.03, imidazole 6.26 ± 0.04 (6.82 ± 0.02), α-amino (6.98 ± 0.04), and phenol 10.2 ± 0.2 (10.5 ± 0.5). The peptide NH–αCH coupling constants in acidic solution are: 6.5 ± 0.3 (Arg), 6.0 ± 0.5, 7.2 ± 0.5, 7.3 ± 0.3 (Phe), 7.0 ± 0.3, and 8.0 ± 0.4. Various classes of labile hydrogens were defined on the basis of their exchange rates as determined from broadening of their respective resonances. The pmr data are consistent either with a rapid equilibrium between various conformations or with a unique conformation of the hormone, but additional evidence is required to definitively determine the structure(s) significantly contributing to the equilibrium. Previously proposed structures excluded by these data include the α helix, the conventional β turn, the γ turn, and a structure stabilized by a salt bridge.

Angiotensin II (AII)[1] is a potent natural pressor agent derived from an α_2-macroglobulin by renin hydrolysis. The present study deals with a more readily available synthetic congener, angiotensinamide (AII)′ (Asn^1-Arg^2-Val^3-Tyr^4-Val^5-His^6-Pro^7-Phe^8), which elicits similar biological responses to AII but differs chemically from it only in the replacement of the N-terminal Asp residue of AII by Asn.

It has long been recognized that the conformation of the hormone may be related to its biological activity (Bumpus *et al.*, 1961). Even though it is the conformation of the hormone at the receptor site which is of primary significance (Marshall and Bosshard, 1972), the free solution orientation of this hormone has received the most attention because it is more easily monitored by available methods. In free solution AII has been variously described as an α helix (Smeby *et al.*, 1962), random-coil (Paiva *et al.*, 1963), antiparallel pleated sheet (Fermandjian *et al.*, 1972a, 1972b; Printz *et al.*, 1972a), and a number of more complex conformations (Weinkam and Jorgensen, 1971a; Glauser *et al.*, 1970). The effect of pH on the conformation of the hormone in free solution has also been debated. Thus, Paiva *et al.* (1963) report no conformational transitions between pH 2.5 and 8.5, but others have associated conformational perturbations with titration of the carboxyl group (Weinkam and Jorgensen, 1971a, 1971b), the imidazole group (Craig *et al.*, 1964), and the phenol group (De Fernandez *et al.*, 1968).

Clarification of this controversy is desirable not only in order to delineate the structure–activity relationship of AII, but also in order to define the capabilities and limitations of various methods employed in studying the conformation of peptides in solution. Toward this end we have undertaken a detailed proton magnetic resonance (pmr) study of the conformation of AII′. A preliminary report of our observations has been presented (Glickson *et al.*, 1972). Here we present a more detailed description of the method of resonance assignment, the effects of pH on conformation, analysis of peptide NH–αCH coupling constants, and estimation of proton exchange rates from line-broadening data.

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[1] Abbreviations used: angiotensin II (AII), and angiotensin II (Asn^1Val^5) (AII′).

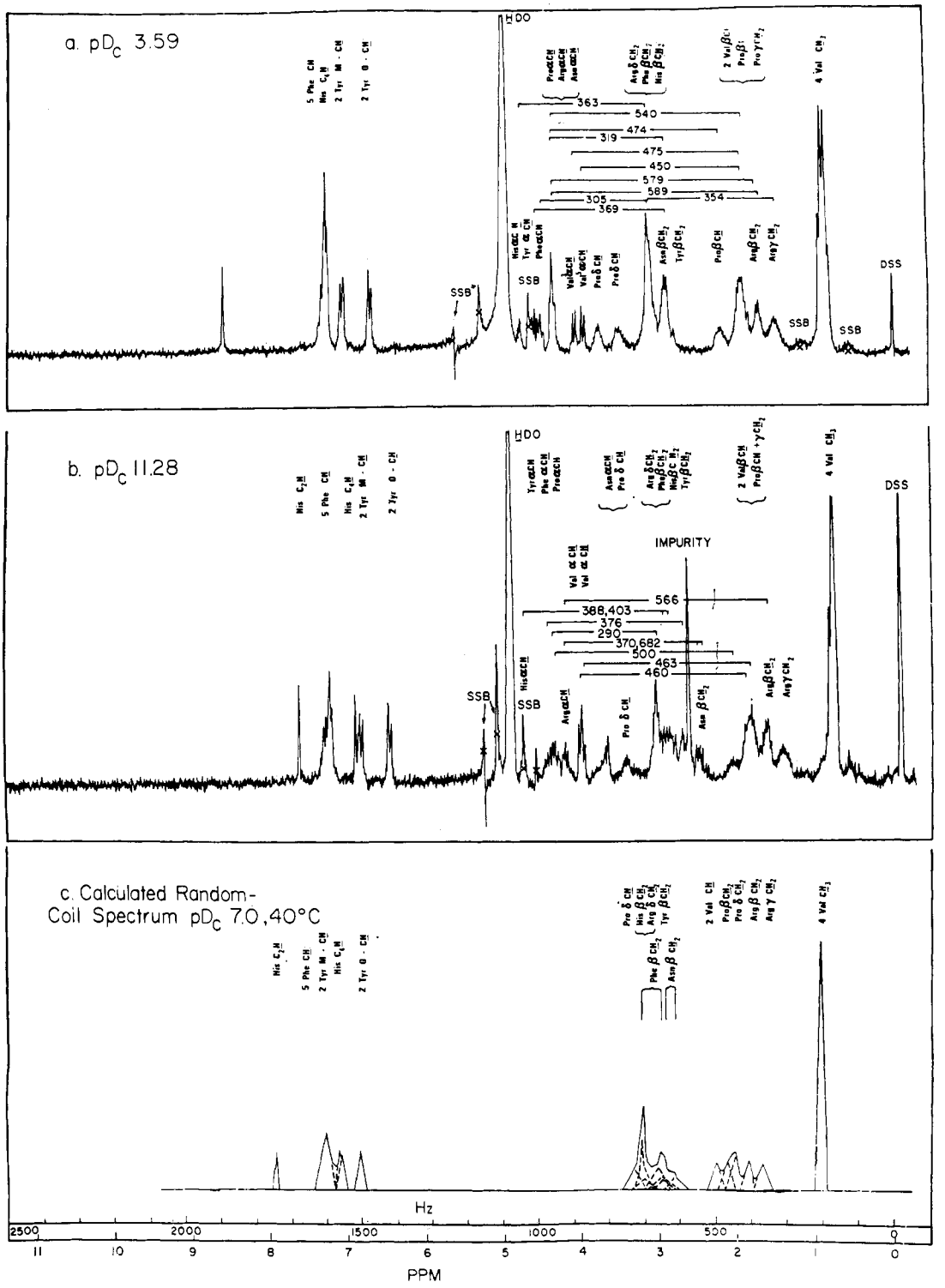


FIGURE 1: Pmr spectra of AII′ 6% (w/v) in D_2O (5 ± 2°) at (a) pD_c 3.59 and (b) pD_c 11.28. Brackets join resonances that were demonstrated to be coupled by double resonance experiments employing the indicated decoupling frequencies. The calculated spectrum for random-coil AII′ in D_2O at pD_c 7.0 and 40° appears in part (c).

Materials and Methods

Angiotensin II (Asn^1Val^5), a generous gift from Ciba Pharmaceuticals (Basel), was used without further purification, except in those experiments requiring removal of trace quantitites of acetate buffer, whose resonance obscured the spectrum. These impurities were removed by ultrafiltration of the hormone against distilled water using a UM-05 membrane (Amicon Corp., Lexington, Mass.). Six per cent (w/v) solutions of AII′ were generally employed except when the sample precipitated near neutral pH. In this pH range the precipitate was removed by centrifugation and measurements were made on the soluble phase. An analog of AII′ containing a [^{2}H]Val residue in position 5 was prepared by solid-phase synthesis (Marshall, 1970). The pD_c was the pH meter reading +0.40. The pH (pD_c) was adjusted with NaOH and HCl (or their deuterated analogs). No correction for sodium error, which was appreciable above pH 10.0, was applied. Spectra were measured on a Varian Associates HR-

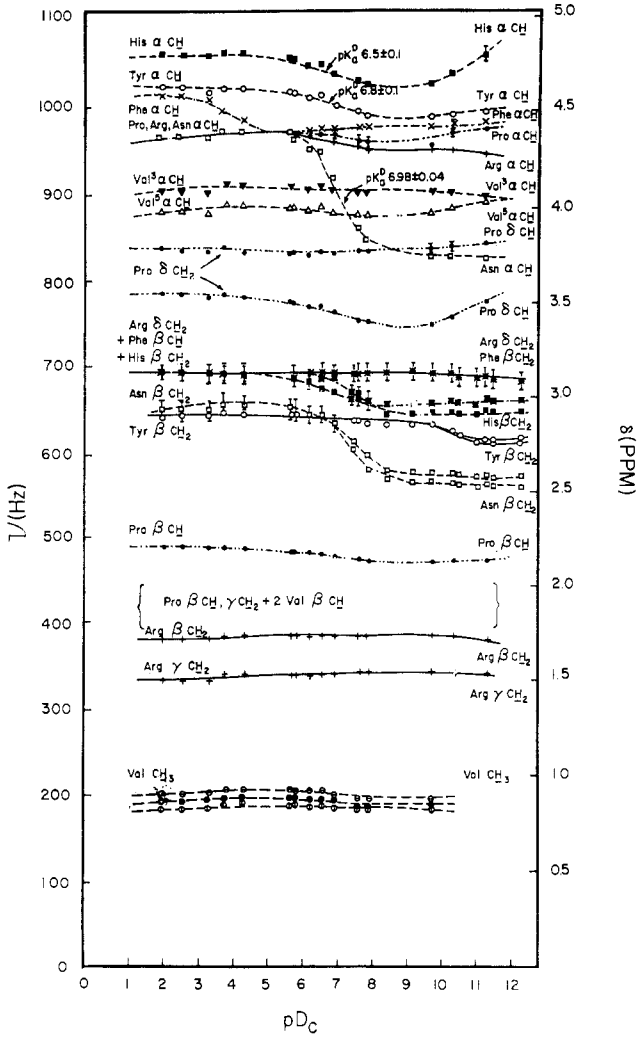


FIGURE 2: Dependence on pD_c of chemical shifts of AII′ resonances which appeared to high field of the HDO absorption at 5 ± 2°. The Pro βCH, γ CH_2, and the two Val βCH absorptions were not adequately resolved to permit accurate tracking of their positions.

220 spectrometer equipped with a 620i computer for signal to noise improvement by multiscan averaging. The normal field sweep decoupling procedure was modified, as described by Walter *et al.* (1972), to simulate frequency sweep decoupling. All chemicals shifts are on the δ scale relative to the methyl resonance of sodium 2,2-dimethyl-2-silapentane-5-sulfonate, the internal standard.

Results and Discussion

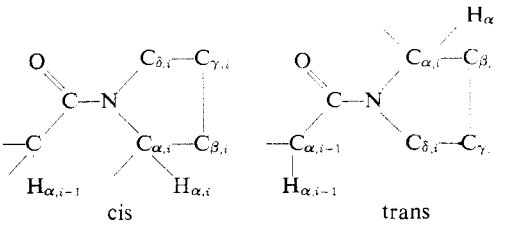
Assignment of Resonances. Pmr spectra of AII′ in acidic and basic D_2O solution appear in Figure 1a,b, respectively. Measurements were made near the solvent freezing point in order to observe all eight of the αCH resonances. At higher temperatures some of these peaks were obscured by the intense residual HDO absorption.

As a starting point for the assignment of resonances to specific hydrogens and for subsequent conformational analysis, we employed the algorithm of McDonald and Phillips (1969) to compute the spectrum of the random-coil hormone shown in Figure 1c. This spectrum is anticipated also for other conformations of AII′ in which all the side-chain hydrogens experience an essentially hydrated environment. Resonances of the α hydrogens, which occur in the 4.0–5.0-ppm region of the spectrum, are not included in this figure because their position depends on sequence position and other factors in a manner which is at present too complex to predict.

The distinct doublets at 3.97 and 4.09 ppm in the αCH region of the spectrum of AII′ in acidic solution (Figure 1a) must originate from α hydrogens of the two valine residues, since this is the only component amino acid with a single β hydrogen. The two Val α-hydrogen resonances were distinguished from each other by specific replacement of the Val-5 hydrogens with deuterium. The 4.09-ppm doublet was absent from the spectrum of the specifically deuterated analog, whereas the 3.97-ppm doublet was unchanged. By spin decoupling we identified the remaining Val resonances.

The random-coil spectrum (Figure 1c) indicates that in addition to the already identified Val resonances, the 1.5–2.5-ppm region of the spectrum contains resonances from the side chains of Arg and Pro. Comparison with the random-coil spectrum suggests that the βCH_2 and γCH_2 groups of Arg are associated with two proton resonances at 1.71 and 1.52 ppm, respectively. This assignment is supported by the demonstrated coupling of the 1.71-ppm resonance to an αCH absorption at 4.36 ppm and coupling of the 1.52-ppm resonance to a resonance at 3.13 ppm, where the Arg δCH_2 peak is anticipated (Figure 1c). The single proton resonance at 2.21 ppm (Figure 1a) which is demonstrated to be a βCH absorption by decoupling from the triply degenerate αCH peak at 4.36 ppm, must therefore originate from Pro, the only component amino acid with an unidentified βCH resonance in this region of the spectrum. The remaining Pro βCH was located in the five-proton multiplet centered at 1.92 ppm by demonstrating that this resonance was also coupled to the Pro αCH peak at 4.36 ppm. A similar magnetic anisotrophy of Pro βCH hydrogens has been observed in spectra of the free amino acid (McDonald and Phillips, 1969). Since Pro γCH_2 absorptions are the only remaining unidentified resonances in this spectral region, they may be assigned together with the two Val βCH peaks, and one Pro βCH peak to the 1.92-ppm five-proton multiplet.

Restricted rotation about the His–Pro peptide bond gives rise to cis and trans isomers. Torchia and Bovey (1971) report



that in aqueous solution the αCH peak of poly(L-proline I), which exists in the cis orientation, occurs at 4.4 ppm, whereas the trans polymer, poly(L-proline II), has an αCH absorption at 4.7 ppm. The 4.36-ppm chemical shift of the Pro αCH peak of AII′ suggests an anomalous cis orientation for peptide bond, however, additional studies would be desirable to determine how reliably the Pro αCH chemical shift distinguishes between cis and trans configurations.

The characteristic pD_c dependence of resonances originating from hydrogens in close proximity to sites of ionization serves as an additional guide to resonance assignment (Figure 2). Thus, the αCH peak of the C-terminal Phe residue was readily identified by its $pK_a = 3.07 \pm 0.03$. Decoupling of this resonance identified the Phe βCH_2 absorption. The

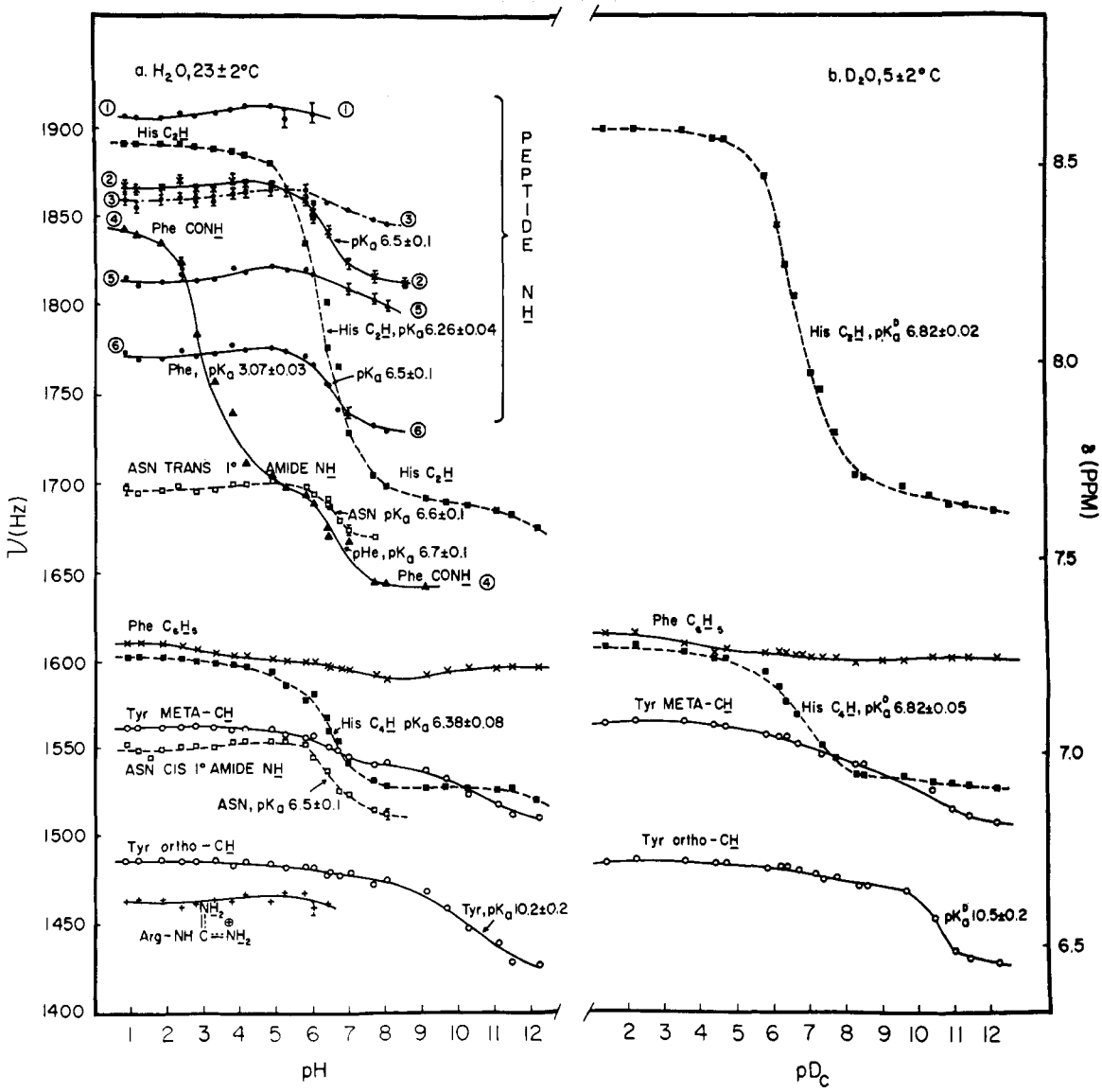


FIGURE 3: (a) Variation with pH of chemical shifts of resonances appearing to low field of the H_2O absorption at 23 ± 2°. The numbers designate peptide NH doublets of which only the one originating from C-terminal Phe has been identified. (b) A similar plot of low field resonances in D_2O at 5 ± 2° after complete exchange of labile hydrogens.

most pD_c-dependent αCH peak is identified by its pK_a = 6.98 ± 0.04 with either the His or Asn residues. The latter assignment is favored both because the magnitude of the spectral changes suggests close proximity to the titration site (the Asn αCH hydrogen is two atoms removed from the N-terminal amino group, whereas the His αCH hydrogen is four atoms from the nearest imidazole nitrogen) and because a significantly lower pK_a = 6.82 ± 0.02 was determined from displacement of imidazole C_2H and C_4H peaks (Figure 3).

The aliphatic absorptions of His and Tyr residues were distinguished by the contrasting pD_c dependence of their βCH_2 absorptions. The Tyr βCH_2 peak shifted to high field in the region of phenol titration, whereas the His βCH_2 peak underwent a similar shift in the imidazole titration range (Figure 2). For purposes of identification of resonances associated with residues other than those at the C terminus or N terminus the pD_c dependence of αCH resonances proved less reliable than the βCH_2 resonances because the αCH hydrogens are influenced by changes in backbone conformation, and because they are further removed from sites of ionization on amino acid side chains. The His and Tyr αCH resonances were therefore identified by double irradiation of their corresponding βCH_2 absorptions.

The Pro δCH resonances at 3.53 and 3.55 ppm were identified by exclusion, once all the remaining aliphatic absorptions were assigned. Spin decoupling experiments with these resonances proved unfeasible as a result of the broadness of the δCH absorptions and overlap of the γCH_2 peaks with Val and Pro βCH absorptions.

Identification of aromatic CH resonances was accomplished in a straightforward manner from their characteristic chemical shifts (Figure 1c), from the unique pD_c dependence of His and Tyr absorptions, from spectral intensities (Figure 3), and from identifiable spin coupling patterns (Figure 1).

Most pmr studies are performed in D_2O in order to minimize the intensity of solvent absorptions. In this solvent resonances associated with labile hydrogens, such as those bonded to nitrogen, do not appear because these protons are rapidly replaced by deuterium upon dissolving the peptide. In H_2O solution, however, resonances of labile hydrogens

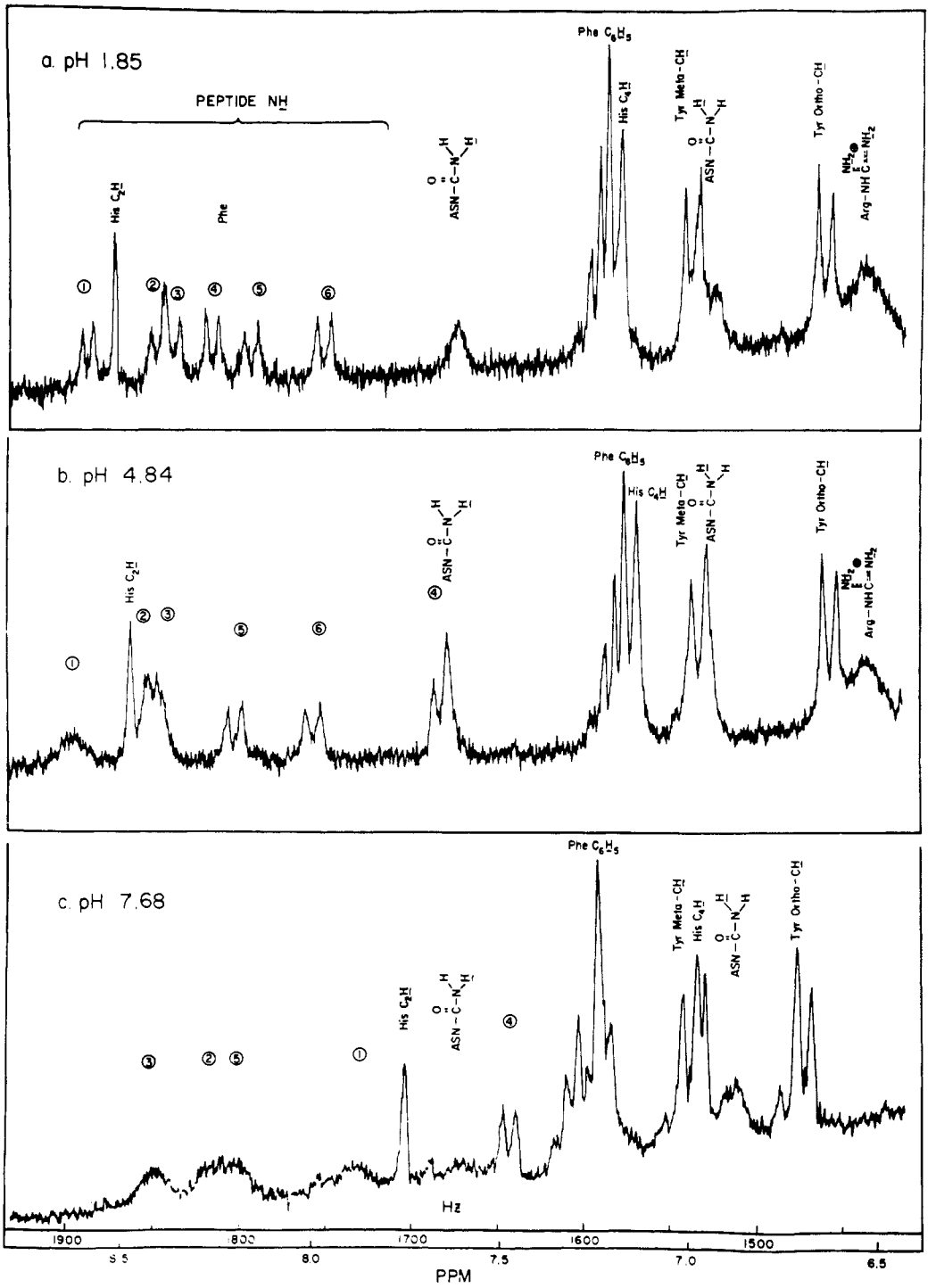


FIGURE 4: The region of the pmr spectrum of AII′ to low field of the solvent absorption in H_2O (23 ± 2°) at pH (a) 1.85, (b) 4.84, and (c) 7.68.

that do not exchange with the solvent at a prohibitively rapid rate are observed (Figure 4). Thus, restricted rotation about the primary amide CN bond of Asn produces distinct resonances at 6.82 and 7.70 ppm corresponding to amide hydrogens cis and trans to the carbonyl oxygen, respectively (Anet and Bourn, 1965) (Figure 4a). Six doublets, each of one proton intensity, originate from peptide hydrogens, of which only the Phe resonance (no. 4 in Figure 4) was unambiguously identified by the characteristic pH dependence of a C-terminal peptide absorption (Figures 3 and 4). The C-terminal Gly NH peak of Gly-Ala-Gly behaved similarly (pK_a = 3.5 *vs.* 3.07 ± 0.03 for AII′).

A four proton resonance at 6.53 ppm (Figure 4) was identified with the four hydrogens of the protonated Arg guanidino group which are equivalent by virtue of the following resonance structures. Poly(L-arginine) had a similar guanidino resonance at 6.64 ppm.

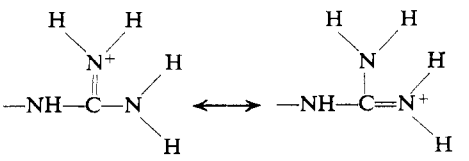


TABLE I: Dissociation Constants of AII′.

Residue	Group	Method of Observation: Pmr[a] pK_a	Pmr[a] pK_a^D	^{13}C Nmr[b] pK_a^D	Direct Titration[c] pK_a
Phe[8]	Carboxyl	3.07 ± 0.03		3.50 ± 0.1	3.34
His[6]	Imidazole	6.26 ± 0.04	6.82 ± 0.02	6.94 ± 0.1	6.30
Asn[1]	α-Amino		6.98 ± 0.04		6.92
Tyr[4]	Phenol	10.2 ± 0.2	10.5 ± 0.2	10.85 ± 0.1	10.04

[a] Carboxyl, imidazole, α-amino, and phenol titrations were monitored by displacement of Phe peptide NH, imidazole C_2H and C_4H, Asn αCH, and Tyr *o*-CH resonances, respectively. Measurements of pK_a's were made in H_2O at 23° and of pK_a^D's in D_2O at 4°. [b] Data of Zimmer *et al.* (1972) in D_2O at 27°. A factor of 0.40 has been added to values reported by these authors to correct for the deuterium isotope effect. [c] Data of Paiva *et al.* (1963) at 25° in 0.1 M KCl.

Acid–Base Titration. Paiva *et al.* (1963) measured the dissociation constants of AII′ by direct titration. Table I compares their data with our own estimates obtained by pmr spectroscopy and with values obtained by Zimmer *et al.* (1972) employing ^{13}C nmr spectroscopy. The unique ability of nmr spectroscopy to monitor the ionization of specific groups of the molecule (Figures 2 and 3) greatly simplifies interpretation of the complex overall titration curve.

Comparison of the various data is complicated by differences in experimental conditions, solvent and temperature. The pmr experiments in H_2O and the direct titration were performed under similar conditions and these data agree quite closely except with respect to the carboxyl group. Errors introduced by the complex curve-fitting procedure employed in extracting dissociation constants from the direct titration curve could account for this discrepancy. Korman and LaMer (1936) measured an increase in the dissociation constants of acetic acid of 0.50 unit on going from H_2O to D_2O. Assumption of a similar deuterium isotope effect for the carboxyl group of AII′ places the dissociation constant determined by pmr spectroscopy in H_2O (3.07 ± 0.03) in good agreement with the value determined by Zimmer *et al.* (1972) by ^{13}C nmr spectroscopy in D_2O (3.50 ± 0.01).

With the exception of the guanidino group, whose pK_a was not measured because of the high sodium error encountered at high pH, the pK_a values of all the ionizable groups were in the range expected for solvated ionizable groups (Greenstein and Winitz, 1961). That this is true even for the apparently low pK_a of the α-amino group can be demonstrated by the following data taken from Greenstein and Winitz's compilation (1961, p 488). The pK_a of Asn-Gly (7.21) is 0.96 p*K* unit below that of Gly-Gly (8.17). Extension of Gly-Gly to $(Gly)_6$ lowers the pK_a = 0.57 unit to 7.60. Assumption of a similar decrease in pK_a for elongation of Asn-Gly yields an estimated value of 6.64 for the pK_a of the α-amino group of Asn-$(Gly)_5$. Hence the low pK_a of the α-amino group of AII′ is probably normal for an N-terminal Asn residue. The normal pK_a values of the ionizable groups of AII′ suggests that they are all hydrated. This, however, does not preclude a folded backbone conformation.

Availability of data on the pH dependence of pmr and ^{13}C nmr spectra (Zimmer *et al.*, 1972) of AII′ permits comparison of the relative sensitivity of these techniques to ionization and conformational equilibria. The ^{13}C nmr titration curves of Zimmer *et al.* (1972) indicate that only resonances of carbon atoms proximal to ionizing sites of AII′ experience substantial changes in their chemical shifts. These perturbations arise from charge density changes associated with the ionization (Horsley and Sternlicht, 1968). There is no hint of any conformational transition. Chemical shifts of pmr resonances are sensitive to both ionic and conformational equilibria since perturbations are observed of resonances associated with hydrogens both proximal to and remote from the titration site. For example, near neutral pH perturbation of Asn and His resonances can be attributed, at least in part, to charge density changes associated with dissociation of ammonium and imidazolium cations. However, substantial displacement of other resonances signifies a conformational transition, *e.g.*, Tyr αCH. Pro δ (Figure 2), Asn cis and trans primary amide NH, Tyr *m*-CH, and peptide NH 2, 3, 4 (Phe), 5, and 6 (Figure 3). That the high field shift of NH resonances did not result from base catalyzed proton exchange with the solvent was demonstrated by studies with asparagine, tryptophan, poly(L-lysine), and poly(L-arginine) (J. D. Glickson and W. D. Phillips, unpublished data) which showed that as the pH was increased resonances of labile hydrogens broadened out but experienced no change in chemical shift. Only when solute concentrations approach that of the solvent is exchange broadening accompanied by coalescence of NH and H_2O resonances. The conformational transition, first reported by Craig and associates (1964) on the basis of their thin-film dialysis studies, has a pK_a of 6.6 ± 0.2 (in H_2O), which suggests involvement of both the α-amino and imidazole groups. This transition is rapid on the nmr time scale with first-order rate constant no less than 50 sec^{-1} (Pople *et al.*, 1959).

In the region of phenol titration, the pmr spectrum indicates another conformational transition by displacement of resonances originating from the His αCH, Pro δCH (Figure 2), and Val CH_3 hydrogens. The positions of overlapping Val CH_3 resonances could not be traced throughout this titration but distortion of their complex envelope gave ample evidence of a transition. By thin-film dialysis De Fernandez *et al.* (1968) noted that titration of the phenol group is accompanied by a marked expansion of AII′.

While chemical shifts of pmr resonances are more sensitive to conformational transitions than chemical shifts of ^{13}C nmr resonances, the latter appear more sensitive to ionic equilibria. Thus, the spectral changes observed by Zimmer *et al.* (1972) are in the order of 1–2 ppm, whereas pmr spectral changes of resonances associated with hydrogens close to the titration site were generally less than 0.5 ppm. The largest pmr change, that of the His C_2H resonance, was about 1 ppm, but this includes a partial contribution from a con-

TABLE II: Peptide NH–αCH Coupling Constants for γ-Turn and β-Turn Models of AII'.[a]

	γ Turn		β Turn	
Residue	θ[b] (deg)	J (Hz)	θ (deg)	J (Hz)
Asn[1]				
Arg[2]	175	9.4	168	9.1
Val[3]	115	3.2	164	8.9
Tyr[4]	7	6.3	5	6.3
Val[5]	168	9.1	158	8.4
His[6]	132	5.3	144	6.9
Pro[7]	120		120	
Phe[8]	127	4.7	147	7.3

[a] From structures proposed by Printz *et al.* (1972a). [b] These angles are defined by Ramachandran *et al.* (1971).

formational change. The greater sensitivity of ^{13}C chemical shifts to ionic equilibira may arise in part because there usually is a carbon atom in closer proximity to a center of dissociation than a hydrogen atom whose resonance can be observed, *e.g.*, the α carbon of Phe is closer to the C-terminal carboxyl oxygen than is either the α hydrogen or the peptide hydrogen.

The nmr studies of AII' illustrate that both ^{13}C nmr and pmr spectroscopy greatly simplify the interpretation of complex titration equilibria. The pmr spectrum gives additional information about the pH dependence of the conformation of the molecule, although this information may also be determined by ^{13}C nmr relaxation studies. Dissociation constants are most easily determined from the ^{13}C nmr spectrum both because of the greater sensitivity of this technique to ionization equilibrium and because of the absence of complications associated with conformational transitions.

Weinkam and Jorgensen (1971a) have suggested that an ionic bond is formed between the protonated imidazole group and the C-terminal carboxylate ion of AII'. The absence of perturbations in the His C_2H and C_4H titration curves in the region of carboxyl group titration (Figure 3) argues against such a bond, which would be disrupted upon protonation of the carboxyl group.

Coupling Constants. Barfield and Karplus (1969) have shown that the peptide NH–αCH coupling constant J depends on the dihedral angle θ of the HNCH fragment in the following manner.

$$J = A\cos^2\theta + B\cos\theta + C\sin^2\theta \qquad (1)$$

where A, B, and C are constants whose values have been empirically estimated as $A = 7.9$ Hz, $B = -1.55$ Hz, and $C = 1.35$ Hz (Ramachandran *et al.*, 1971). Equation 1 permits evaluation of various models which have been proposed for the preferred solution conformation of AII' in terms of the observed values of J, which are 6.5 ± 0.3, 6.0 ± 0.5, 7.2 ± 0.5, 7.3 ± 0.3 (Phe), 7.9 ± 0.3, and 8.0 ± 0.4 Hz for peptide resonances 1–6, respectively, in acidic solution (Figure 4). Because these coupling constants are averaged over all the conformations of the molecule, a strict quantitative interpretation of J in terms of eq 1 is not possible, if more than one conformation contributes significantly to the conformational average. Recognizing this limitation, we restrict ourselves to a semiquantitative treatment. It is clear that an α-helical conformation ($J \sim 2$ Hz) such as was suggested by Bumpus *et al.* (1961) cannot make a significant contribution to the conformational equilibrium. Similarly, the observation of small coupling constants (<3 Hz) in pmr spectra of the heptapeptide analog of AII' studied in Me_2SO by Weinkam and Jorgensen (1971a) proves that this analog is a poor model for AII' in aqeuous solution. A random-coil orientation ($J = 6.1$ Hz) (Tonelli and Bovey, 1970), such as has been suggested by Paiva *et al.* (1963), while consistent with two of the observed coupling constants, is inconsistent with the remaining four. Furthermore, the chemical shifts of a number of AII' resonances differed significantly from values predicted for the random-coil peptide (Figure 1), *e.g.*, the Pro δCH_2 peaks at 3.53 and 3.56 ppm (random-coil, 3.35 ppm), and the Val βCH peaks at 1.93 ppm (random-coil, 2.24 ppm). The data are, however, consistent with the suggestion that the hormone is rapidly interconverting between a number of conformations, some of which are largely disordered (Zimmer *et al.*, 1972; Fermandjian *et al.*, 1971).

Printz *et al.* (1972a) and Fermandjian *et al.* (1971, 1972a, 1972b) favor a significant contribution from an antiparallel pleated sheet structure to the conformational equilibrium of AII' in aqueous solution. Table II summarizes the peptide NH–αCH coupling constants anticipated for two of the structures proposed by Printz *et al.* (1972a). The γ turn, the structure they favor most strongly, can be excluded as a significant structure since it requires three coupling constants to be well below the range observed in the pmr spectrum. Their β-turn model is consistent with the observed coupling constants. However, this model presumes an orientation of the Tyr residue ($\phi = 55°$, $\psi = -99°$) which is opposite to that predicted by potential energy calculation for the $i + 1$th residue of a β turn (Urry and Ohnishi, 1970). Moreover, such an orientation has never been observed in any protein whose structure has been determined by X-ray diffraction. Such a structure while anomalous cannot, however, be excluded. The conventional orientation of the Tyr residue would require a coupling constant (2 Hz) too small to be consistent with observed values.

Proton Exchange Rates. The rate at which a labile hydrogen exchanges with atoms of the solvent is often a measure of its accessibility to the solvent. Conventional methods of monitoring proton exchange rates have led to conflicting conclusions that AII' is a random-coil (Paiva *et al.*, 1963) and that it exists in a unique folded conformation (Printz *et al.*, 1972b). This ambiguity may result from the fact that conventional methods monitor the simultaneous exchange of all labile hydrogens, and interpretation of the complex kinetics in terms of individual exchange rates is often a difficult process. We have therefore reexamined the proton exchange kinetics of AII' by pmr spectroscopy, a technique uniquely capable of directly monitoring the exchange of individual hydrogens. Resonances of NH hydrogens broaden out when the pseudo-first-order rate constant associated with the exchange of these hydrogens with protons of the solvent becomes comparable to the difference between the chemical shifts of resonances associated with the exchanging protons (typically about 1000 sec^{-1}). If allowances are made for small differences between the chemical shifts of NH resonances, a semiquantitative measure of their exchange rates, sufficiently accurate for the purposes of the present study, is the pH at which these NH resonances broaden out, the smaller this pH, the more rapid the exchange. Significant differences were observed in the pH at which various NH resonances broadened (Figure 4). The labile hydrogens of AII' fall into four distinct kinetic groups:

group I consists of the α-NH_3 and the unique guanidino proton ($NHC(NH_2)=NH_2$) all of which exchange too rapidly to be observed. The next most rapidly exchanging protons comprising group II are peptide 1 and the four equivalent Arg-($NHC(NH_2)=NH_2$) protons, whose resonances broaden out between pH 5 and 6. Group III consists of the primary amide NH absorptions and all the remaining peptide resonances except for the Phe peptide NH peak. Resonances in this group broaden out between pH 7 and 8. The most slowly exchanging NH hydrogen (group IV) originates from the C-terminal Phe peptide proton, whose resonance does not broaden out until pH 10. Since a negatively charged C-terminal group is expected to destabilize the amide anion transition state for proton exchange (Berger *et al.*, 1959), an anomalously slow exchange rate would be anticipated for the C-terminal peptide on purely inductive grounds. This conclusion is supported by the strikingly similar behavior of the C-terminal Gly peptide resonance of Gly-Ala-Gly. Conversely, the positively charged N-terminal amino group and the Arg guanidino group would be expected to enhance the exchange rate of the Arg peptide hydrogen. This suggests that peptide resonance 1 originates from Arg. A more rigorous kinetic analysis of exchange kinetics from line broadening data requires complex curve fitting procedures (Johnson, 1965; Binsch, 1969).

Intermolecular Interactions. By sedimentation studies Paiva *et al.* (1963) demonstrated that AII′ remains in the monomeric state up to concentrations as high as 1% (w/v). Precipitation of the hormone near neutral pH demonstrates that intermolecular aggregates can form once the amino and/or the imidazole groups are discharged. However, it appears that the remaining soluble hormone remains predominantly in the monomeric form. Thus, no concentration dependence of the pmr spectrum was observed between 5×10^{-2} and 10^{-3} M, and the observed line width of 1 Hz or less is that expected of the monomer.

Conclusions

Observation of no more than one resonance for every hydrogen of AII′ indicates that in aqueous solution this hormone either assumes a unique orientation, or else an equilibrium, rapid on the nmr time scale, exists between various conformers. Additional analysis of nmr data may help to definitively distinguish between these alternatives.

Since all the peptide NH–αCH couplings are large, the implications to be drawn from these parameters can to a large measure be drawn even without a complete assignment. Thus, all conformations which require at least one small peptide NH–αCH coupling constant can at once be eliminated as significant contributors to the conformational equilibrium. This excludes the left-handed α helix, the γ turn, and the β turn with the usual orientation of the corner residue. The β turn suggested by Printz *et al.* (1972a) in which an anomalous orientation is assumed by the Tyr residue is, however, consistent with the pmr data, although it conflicts with potential energy calculations. It may be anticipated that other consistent cross-β structures can also be constructed. However, a soluble intermolecular β structure, while consistent with observed couplings, appears unlikely on the basis of evidence for limited aggregation in the aqueous phase. The absence of small peptide NH–αCH coupling constants and the limited extent of spectral perturbations associated with titration of the carboxyl group argue against the structure proposed by Weinkam and Jorgensen (1971) in which a salt bridge was assumed between the C-terminal carboxylate anion and the imidazole cation. A completely disordered orientation appears unlikely in the light of distinct deviations from the random-coil spectrum.

All the nmr evidence is fully consistent with the proposal that a dynamic equilibrium exists between disordered forms of AII′, and various ordered structures, of which the most prevalent is an antiparallel pleated sheet (Fermandjian *et al.*, 1971, 1972a, 1972b). However, at this stage it is premature to draw definitive conclusions about the preferred solution conformation of AII′. The present study has been directed primarily in laying the foundation for subsequent pmr studies of this hormone. Much additional information remains to be extracted from the pmr spectrum.

The nature of the conformational changes remains to be elucidated. Preliminary experiments indicate that addition of guanidine–DCl to AII′ at pD_c 9.4 does not perturb the pmr spectrum of the hormone, but at pD_c 4.4 some alterations are observed. This suggests that in the transition which occurs near neutral pH an alkaline medium favors a more disordered structure. The data of De Fernandez *et al.* (1968) suggest that a further unfolding of the hormone accompanies ionization of the phenol group.

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Purification and Characterization of a Steroid Receptor from Chick Embryo Liver†

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ABSTRACT: The high-speed supernatant fraction of the 16-day-old chick embryo liver homogenate was found to contain a macromolecular species that could form tight complexes with steroid hormones. This hormone receptor was purified by isoelectric precipitation, high-speed centrifugation, DEAE-cellulose column chromatography, and preparative disc electrophoresis. The purified receptor migrated as one diffuse band on analytical acrylamide gel electrophoresis and had an isoelectric point of 5.3, as shown by electrofocusing. The hormone–receptor complex sedimented as a single 4S species in sucrose gradient centrifugation in several concentrations of KCl. The binding of hydrocortisone to the receptor has a temperature optimum at 37°, and saturation is reached within 10 min of incubation. The binding was insensitive to changes in pH within the range of 4.5–9.4. By the use of equilibrium dialysis it was estimated that the association constant for the binding of hydrocortisone was 7.5×10^8 M^{-1} at 4°, while that for the binding of corticosterone was 3.6×10^8 M^{-1} at 4°. The protein nature of the receptor was suggested by its susceptibility to proteolytic enzymes. Estradiol and testosterone were found to bind to the crude receptor preparation (the high-speed supernate). This binding, however, would not reach saturation even when high concentrations of hormones were used. In addition, neither estradiol nor testosterone would compete with the binding of hydrocortisone or corticosterone by the receptor. After the receptor was purified by preparative disc electrophoresis, the binding of hydrocortisone and corticosterone was at least 3000-fold higher than that of estradiol and testosterone, when compared in the range where the binding of hydrocortisone was linear with respect to hormone concentrations.

Several approaches have been used for the elucidation of the mechanism of hormone action. Injection of radioactive hormone into animals showed concentration of radioactivity in specific target organs (Bellamy *et al.*, 1962; Litwack and Baserga, 1967). Induction of RNA and protein synthesis after *in vivo* injection of hormone into an animal has also been demonstrated (Kenney, 1962; Schimke *et al.*, 1965; Teng and Hamilton, 1968).

The specificity of action manifested by the hormone is incompatible with its being a low molecular weight substance. It has been hypothesized (Jensen and Jacobson, 1960) that the target tissue may play an important role in the final physiological manifestation of hormone action. More recently, receptor molecules for specific hormones have been isolated from their target organs. Such a receptor was initially isolated from the rat or calf uterus, which bound estrogen hormones (Toft and Gorski, 1966; Jensen *et al.*, 1967; Erdos, 1968; Puca *et al.*, 1971). The hormone–receptor complex was isolated from the soluble supernate of the uterine homogenate and was found to sediment as an 8S molecule on sucrose gradient. This 8S molecule dissociated into a 4–5S species upon treatment with 0.3 M KCl. Similar receptor molecules have been reported for other steroid hormones (Edelman and Fimognari, 1968; Gardner and Tomkins, 1969; Sherman *et al.*, 1970). There is evidence that these receptor molecules enter the nucleus of the target tissue in the presence of hor-

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