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Association of Sexual Hormones with Nucleobases in Water: NMR-Investigations

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Abstract. The mixed association of testosterone-sulfate and estradiol-sulfate with several derivatives of nucleobases in D_2O has been investigated by means of nuclear magnetic resonance spectroscopy. From the differences among the chemical shifts of the hormone-protons it is concluded, that the nucleobases in the complexes are located above the center of the steroid molecule. The β-side of the steroid which is characterized by the axial methyl-groups is directed towards the bases. The enthalpies of mixed association of the hormones with a certain nucleobase are of the same order of magnitude as the enthalpy of selfassociation of this nucleobase (Schimmack *et al.*, to be published). It is suggested that the complexes are stabilized by van-der-Waals forces. This stacking-like interaction is not specific for the male or female sex hormones: no qualitative or quantitative differences have been observed among the complexes of the two hormone-sulfates with the nucleobases.

Key words: Sexual Hormones — Nucleobases — NMR — Stacking — Enthalpies

Introduction

The molecular action of the sexual hormones is still unknown. There is a lot of experimental evidence that the sex hormones stimulate the biosynthesis of ribonucleic acid (RNA) and proteins in the nucleus (O'Malley and Means, 1974). It seems to be well established that, in vitro, the sex hormones bind reversibly to nucleic acids, especially to single-stranded parts of the deoxyribonucleic acid (DNA) (Ts'o and Lu, 1964) and to aminoacylated tRNAs (Chin and Kidson, 1971). Since covalent and electrostatic binding can be excluded, the association must be due to weak noncovalent interactions like hydrogen bonding, charge-transfer, and van-der-Waals interaction.

Recently we have shown that the sexual hormones form hydrogen bonds to nucleobases in chloroform (Schimmack, 1975; Schimmack and Lohmann, 1973). In the present study nuclear magnetic resonance (NMR-) experiments have been conducted in order to determine the interaction between water-soluble derivatives of sex hormones and nucleobases in D_2O. In order to elucidate the mechanism of action the type and loci of this interaction as well as the differences between the sexual hormones or the nucleobases have been investigated.

These investigations may contribute to the discussion of the models proposed for the molecular interaction between sexual hormones and nucleic acids. Moreover, the results may be useful for a better understanding of the association of sexual hormones with proteins.

Materials and Methods

In this study, two water-soluble hormone-derivatives have been used: estradiol-3-sulfate (ES) and testosterone-17-sulfate (TS) (s. Fig. 1).

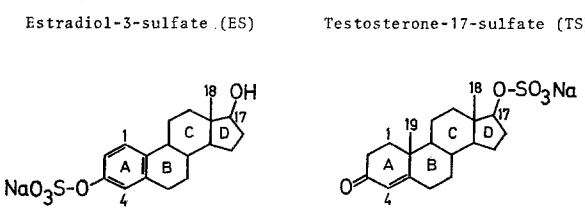

Fig. 1. The water-soluble hormone derivatives

ES has been synthesized as follows: The pyridine-salt of estrone-sulfate was prepared by using the estrone and pyridine/SO_3-complex. Then, this compound was transferred into the Na-salt with sodium-methylate. Addition of $NaBH_4$ resulted in the Na-salt of estradiol-3-sulfate which was purified and recristallized several times.

TS has been purchased from Merck-Schuchardt, Darmstadt, Germany, and used without further purification.

The purine- and pyrimidine-derivatives used in this study are abbreviated as "nucleobases" or, simply, as "bases". N^9-ethyl-adenine (e^9Ade), N^6,N^9-dimethyl-adenine (m^6m^9Ade), N^6-dimethyl-N^9-ethyl-adenine ($m_2^6e^9$Ade), and N^9-ethyl-hypoxanthine (e^9Hyp) have been purchased from Cyclo Chemical, Los Angeles, USA, N^6-dimethyl-adenine (m_2^6Ade) and N^6-dimethyl-adenosine (m_2^6Ado) from Merck-Schuchardt, Darmstadt, Germany, and Coffein, tetramethyl-uric acid (TUA), N^1-methyl-cytosine (m^1Cyt), and N^1,N^3-dimethyl-uracil (m^1m^3Ura) from Fluka, Buchs, Switzerland. Coffein was recristallized from H_2O, the other nucleobases were used without further purification. D_2O, tertiary butanol (tB), and 2,2,3,3-tetradeutero-3-(trimethylsilyl)-propionic acid Na-salt (TPA) have been obtained from Merck-Schuchardt.

All NMR-spectra have been recorded on a Varian-HA-100 spectrometer equipped with a variable temperature system. The temparetures were calculated according to a calibration proposed by Van Geet (1968 and 1970). All chemical shifts were measured relative to TPA (zero point of the δ-scale) measured in a separate probe.

The thermodynamic quantities of selfassociation were calculated according to the isodesmic NMR-model described elsewhere (Schimmack *et al.*, 1975). This model was extended in order to calculate the thermodynamic quantities of mixed association. The models of Antonovsky *et al.* (1973) and of Dimicoli and Hélène (1973) are special cases of this model as has been discussed in the case of self-association (Schimmack *et al.*, 1975).

The method for calculating the apparent equilibrium constants of mixed association K_c (at 25° C: $K_c^{25\,C}$) is described in the appendix. The temperature dependence of these K_c-values was used for calculating the enthalpies ΔH as well as ΔS of the mixed associations according to the Van't Hoff equation.

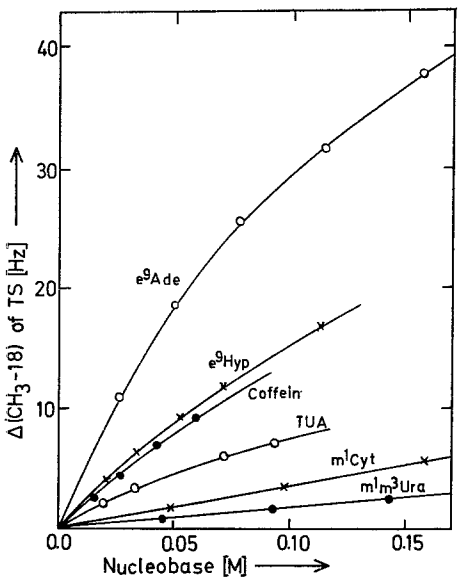

Fig. 2. Mixed association of testosterone-sulfate (TS) with nucleobases in D_2O: upfield shifts of the 18-methyl group of TS. [TS] = 0.02 M. Temperature: ca. 4° C

For the investigation of the mixed associations two sets of experiments have been performed for each hormone. In both cases, the concentration of the bases has been varied (compound A) while the concentration of the hormone has been kept constant (compound B): 0.02 M in the first series and 0.003 M in the second one, in which the methyl-groups of the hormones could be observed only.

The complex shifts Δ_c^B appeared to be temperature dependent. The values $\overline{\Delta_c}$ listed in the table are values averaged over the temperature-interval used.

The errors of the thermodynamic quantities, which have been averaged over all possible combinations of the protons of A and B, are less than $\pm$ 15% for ΔH and $\pm$ 20% for K_c and ΔS, resp.

Results

In solutions of estradiol-sulfate (ES) and testosterone-sulfate (TS) with nucleobases the resonances of all protons of the hormones as well as of the bases are shifted upfield to a varying degree. As an example, the upfield shifts of the CH_3-18-group of TS are shown in Fig. 2 as a function of the concentration of the nucleobases (e^9Ade resembles the behaviour of the 4 other adenine-derivatives used).

There are three "characteristic" resonances in the spectra of the hormones which are suitable both for qualitative considerations and quantitative calculations as well: the resonances of the protons H-4, CH_3-18, and CH_3-19 of TS and H-4, CH_3-18, and H-1 of ES (s. Fig. 1). Comparing the upfield shifts of these resonances in the mixed associates of the hormones with the nucleobases, *e.g.* e^9Ade (s. Fig. 3), it is obvious that the shifts of both hormones are of the same order of magnitude and that the protons 4 and 18 are less influenced than the

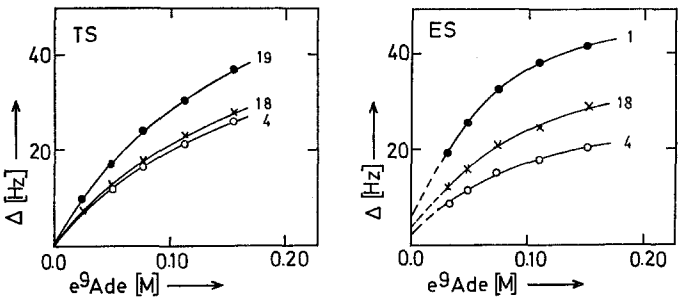

Fig. 3. Mixed association of testosterone-sulfate (TS) and estradiol-sulfate (with e^9Ade in D_2O: upfield shifts of the "characteristic" resonances. [TS] = [ES] = 0.02 M. Temperature: ca. 40° C

protons 1 and 19, respectively. At zero concentration of e^9Ade, the shifts of the ES-protons are not zero due to a slight selfassociation of ES (s. below).

The upfield shifts of the hormone-resonances are caused by the magnetic anisotropy of the nucleobases. The resonances of the base-protons are shifted upfield due to the selfassociation of the bases which is a special case of the stacking-interaction (Ts'o, 1970; Pörschke and Eggers, 1972). In the vertical stacks, the base-protons are shielded due to the ring-current effects of the bases (Ts'o, 1970). When a hormone is added to a base or to an aggregate of bases in a stacking-like way, the protons of the hormone are shielded, too.

The selfassociation of the nucleobases has been discussed in detail elsewhere (Schimmack *et al.*, to be published). From the two hormone-sulfates used, ES only exhibited a small selfassociation: $K_s^{25\,C} = (2.9 \pm 1.4)\ \mathrm{M}^{-1}$ and $-\Delta H = (1.4 \pm 0.7)$ kcal/mol (values averaged over protons H-1 and CH_3-18). Due to this selfassociation and to the magnetic anisotropy of the phenolic A-ring of ES, which is more pronounced than the magnetic anisotropy of the acrolein-like group in the A-ring of TS, the isodesmic NMR-model can be better applied to testosterone-sulfate.

Since the selfassociation of TUA, m^1Cyt, and m^1m^3Ura has not been evaluated quantitatively (Schimmack *et al.*, to be published), the thermodynamic quantities of mixed association of the hormones with these bases could not be calculated. The thermodynamic quantities of the complexes of TS with the other nucleobases investigated are summarized in Table 1 for both series of experiments.

The enthalpies ΔH and the entropies ΔS of mixed association agree very well within the two experiments. The discrepancy in the apparent equilibrium constants K_c may be explained by the presence of complexes $B_1A_nB_1$ in solutions with higher TS-concentrations. Then, the K_c'-values of the two binding sites (see above) should be in the same order of magnitude as the K_c-values of the experimental series with [TS] = 0.003 M.

According to the $K_c^{25\,C}$-values, the binding tendency decreases as follows:

$$m_2^6e^9\text{Ade} > m_2^6\text{Ado} > m^6m^9\text{Ade} \simeq m_2^6\text{Ade} > e^9\text{Hyp} > e^9\text{Ade} > \text{Coffein}.$$

The sequence of the enthalpies, which are approximately the binding energies, differs only slightly from that one obtained for the $K_c^{25\,C}$-values:

$$m_2^6\text{Ade} > m_2^6e^9\text{Ade} \gtrsim m^6m^9\text{Ade} \gtrsim m_2^6\text{Ado} \gtrsim e^9\text{Ade} > e^9\text{Hyp} > \text{Coffein}.$$

Table 1. Mixed association of testosterone-sulfate (TS) with some nucleobases in D_2O: proton-averaged thermodynamic quantities

Base	[TS]							
	0.02 M				0.003 M			
	pD	$K_c^{25\,C}$	$-\Delta H$	$-\Delta S$	*pD*	$K_c^{25\,C}$	$-\Delta H$	$-\Delta S$
e^9Ade	7.7	5.9	4.0	9.8				
m^6m^9Ade	7.7	11.0	4.3	9.5				
$m_2^6e^9$Ade	7.8	21.2	4.5	9.0	7.8	14.2	4.4	9.3
m_2^6Ade	7.7	10.8	5.8	14.7	7.6	8.0	5.5	14.4
m_2^6Ado	7.6	15.4	4.1	8.2				
e^9Hyp	7.3	6.9	3.0	6.1	7.8	2.5	2.8	7.4
Coffein	7.7	4.5	2.4	5.1				

units: $K_c^{25\,C}$ [M^{-1}] ΔH [kcal · mol^{-1}] ΔS [cal · mol^{-1} · $degree^{-1}$]

Table 2. Mixed association of testosterone-sulfate (TS) with some nucleobases in D_2O: complex shifts $\bar{\Delta}_c$[Hz] of the "characteristic" protons of TS. Experimental series with [TS] = 0.02 M

Base	TS		
	Protons		
	H-4	CH_3-18	CH_3-19
e^9Ade	97	137	166
m^6m^9Ade	91	113	132
$m_2^6e^9$Ade	79	108	129
m_2^6Ade	121	141	183
m_2^6Ado	75	95	123
e^9Hyp	49	44	53
Coffein	80	82	81

As can be seen, m_2^6Ade as the exception, exhibits the largest $|\Delta H|$-value, while its $K_c^{25\,C}$-value is rather low.

The complex shifts $\bar{\Delta}_c$ are summarized in Table 2. Within the adenine-derivatives it is

$$\bar{\Delta}_c(CH_3\text{-}19) > \bar{\Delta}_c(CH_3\text{-}18) > \bar{\Delta}_c(H\text{-}4).$$

The complexes formed between estradiol-sulfate (ES) and the nucleobases could not be evaluated to the same extent as in the case of TS due to the magnetic anisotropy and the selfassociation of ES (see above). Therefore, only the complexes of ES with the adenine-derivatives in the experimental series using [ES] = 0.003 M have been treated according to the isodesmic model (s. Table 3). At this low concentration of ES the selfassociation of ES is negligible, but its magnetic anisotropy enhances the errors to a considerable degree. Nevertheless, it is evident that the strength of the complexes of ES is about the same as that one obtained for the complexes of TS. Moreover, the complexes of the two hormone-sulfates behave qualitatively alike in regard to the upfield shifts of the different protons,

Table 3. Mixed association of estradiol-sulfate (ES) with adenine-derivatives in D_2O: thermodynamic quantities of the 18-methyl-group of ES. [ES] = 0.003 M (*pD* 7.8)

Base	ES			
	K_c^{25C}	$-\Delta H$	$-\Delta S$	$\bar{\Delta}_c$
e^9Ade	4.7	3.8	9.6	127
m^6m^9Ade	10.1	4.6	10.7	92
$m_2^6e^9$Ade	14.6	4.7	10.5	110
m_2^6Ade	8.5	5.7	15.0	125
m_2^6Ado	7.6	4.1	9.6	111

units: K_c^{25C} [M^{-1}] ΔH [kcal · mol^{-1}] ΔS [cal · mol^{-1} · $degree^{-1}$] $\bar{\Delta}_c$ [Hz]

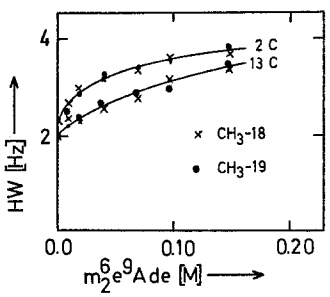

Fig. 4. Mixed association of testosterone-sulfate (TS) with $m_2^6e^9$Ade in D_2O at 2 and 13° C, resp.: half line width (HW) of the resonances of the methyl-groups of TS. [TS] = 0.02 M

especially of the "characteristic" ones, as has been verified in the experimental series using [ES] = 0.02 M.

Discussion

From the results obtained it may be concluded that the association of the sexual hormones to the nucleobases resembles the stacking-interaction exhibited by the nucleobases themselves. This applies to the conformation as well as to the thermodynamic quantities of the complexes.

The conformation of the mixed associates is a vertical stack. From the complex shifts of the three "characteristic" signals of TS (s. Table 2) it is evident that in the complexes formed between TS and the adenine-derivatives, the bases are located above the center of the steroid molecule. In the case of the other nucleobases investigated they are located adjacent to the A-ring. Moreover, from the increase observed in the linewidth of the methyl-groups of TS (*e.g.* in the complexes formed with $m_2^6e^9$Ade, s. Fig. 4) it may be concluded, that the β-side of the steroid, characterized by the two axial methyl-groups, is directed towards the bases.

The assumption of a stacking-like interaction between sexual hormones and nucleobases is supported further by the close relationship between the enthalpies of mixed association, ΔH_M, of the hormones (*e.g.* TS) with the bases and the enthalpies of selfassociation, ΔH_S, of the nucleobases (s. Fig. 5): within the

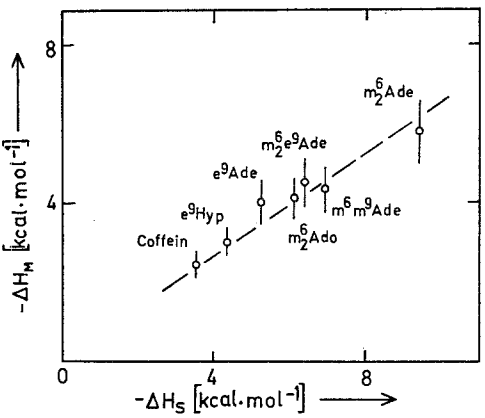

Fig. 5. Relationship between the enthalpies ΔH_M of mixed association of testosterone-sulfate with some nucleobases and the enthalpies ΔH_s of selfassociation of the nucleobases in D_2O. The values of ΔH_s were taken from Schimmack *et al.* (to be published)

experimental errors, ΔH_M is proportional to ΔH_S. The proportionality constant is 0.66 ± 0.08. The attachment of a steroid (mixed association), instead of a base (selfassociation), to a base reduces the binding energy and tendency, but the interaction is still remarkably strong. Antonovsky *et al.* (1973) have measured (by means of NMR-spectroscopy) $K_c^{25\,C} = 6.0\ M^{-1}$ and $-\Delta H = 4.5$ kcal/mol for the stacking association of m^6m^9Ade with m^1m^3Ura. Obviously, these values agree very well with those ones obtained for the complexes formed between the hormone-sulfates and m^6m^9Ade.

The question arises whether the mixed association of the hormones with the nucleobases as well as the selfassociation of the nucleobases are due to the same molecular interaction, *e.g.* stacking interaction. According to the current hypotheses of this interaction (Ts'o, 1970; Pörschke and Eggers, 1972; Lawaczeck and Wagner, 1974), the dipole-induced dipole- and the dispersion-forces stabilize the stacking complex, while the solvent water is the "driving force" of the association. Lawaczeck and Wagner (1974) suggest that the polarizability of the π-electron system and the partial bond moments are more important than the totel polarizability and the total dipole moment.

In regard to the nucleobases, the π-polarizability seems to be essential for the selfassociation of the bases (Lawaczeck and Wagner, 1974; Schimmack *et al.*, to be published) as well as for the mixed association with the hormones (proportionality of the enthalpies). The mixed association seems to be weaker than the self-association of the nucleobases, since the hormones (like the pyrimidine-derivatives) possess in their A-ring only two and three π-electrons, respectively.

Concerning the sexual hormones, the whole steroid molecule seems to be involved in the interaction which might be caused by its total polarizability and/or its polarizing partial bond moments (polar group in the A-ring and, perhaps, the methyl-groups).

In summary it may be concluded, that the sexual hormones interact with nucleobases in water forming stacking-like complexes. The substances are driven

together by the solvent water as has been suggested earlier by Munck *et al.* (1957). The complexes, then, are stabilized, at least partly, by special van-der-Waals forces.

This stacking-like interaction is not specific for the male or female sex hormone: the complexes of testosterone- and estradiol-sulfate with nucleobases are very similar to each other in regard to both qualitative (upfield shifts of the "characteristic" resonances) and quantitative (binding energy and tendency) aspects.

Considering the investigations concerning hydrogen bonds formed between sexual hormones and nucleobases in chloroform (Schimmack, 1975; Schimmack and Lohmann, 1973), it may be suggested, that the molecular mechanism of action of the sexual hormones is based rather on their capability to form hydrogen bonds with nucleobases (and amino-acid residues of proteins) than to participate in stacking-like van-der-Waals interactions.

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Appendix

From the two reacting substances A and B, A only shall be able to selfassociation (equilibrium constant of selfassociation K_s^A). The compound B should be magnetically inert and not be able to selfassociate; its concentration was kept constant within an experimental series in this study. When a monomer B_1 of B is attached to an associate A_n of A ("one-side"-attachment), the complex C_n is obtained:

$$A_n + B_1 \rightleftharpoons C_n \qquad C_n \equiv A_n B_1$$

The equilibrium constant of this mixed association K_c is, then,

$$K_c = \frac{[C_n]}{[A_n] \cdot [B_1]} \qquad n = 1, 2, 3 \ldots$$

$[A_n]$, $[B_1]$, $[C_n]$: concentrations of A_n, B_1, C_n.

With $x \equiv K_s^A \cdot [A_1]$, the initial concentrations $[A_0]$ of A and $[B_0]$ of B in a sample are (Antonovsky *et al.*, 1973):

$$[A_0] = [A_1] \cdot \frac{1 + K_c \cdot [B_1]}{(1 - x)^2}$$

$$[B_0] = [B_1] + \frac{K_c \cdot [A_1] \cdot [B_1]}{1 - x}.$$

x is calculated from the chemical shift $\Delta^A = \delta^A - \delta_M{}^A$ observed for a proton of A ($\delta_M{}^A$: monomer shift). According to the model described recently (Schimmack *et al.*, 1975), the proton has two averaged complex shifts of selfassociation in all associates A_n: the dimer shift $\Delta_2{}^A$ and the trimer shift $\Delta_3{}^A$, both measured relative to δ_M. Using normalized shifts $\alpha \equiv \Delta^A/\Delta_2{}^A$ and $\gamma \equiv \Delta_3{}^A/\Delta_2{}^A$ one obtains

$$x = \frac{\alpha}{1 + \sqrt{1 - (2 - \gamma) \cdot \alpha}}. \tag{1}$$

The monomer concentration $[B_1]$ is obtained from the chemical shift $\Delta^B = \delta^B - \delta_M{}^B$ observed for a proton of B ($\delta_M{}^B$: monomer shift). Assuming an averaged complex shift $\Delta_c{}^B$ of that proton in all complexes C_n and by using normalized shifts $\beta \equiv \Delta^B/\Delta_c{}^B$, it follows

$$[B_1] = [B_0] \cdot (1 - \beta)$$

and

$$\beta = \left[1 + \frac{K_s^A}{K_c} \cdot \left(\frac{1}{x} - 1\right)\right]^{-1} \tag{2}$$

or

$$K_c = K_s{}^A \cdot \frac{\frac{1}{x} - 1}{\frac{1}{\beta} - 1}. \tag{3}$$

If the monomers B_1 are attached to both ends of A_n ($C_n' \equiv B_1 A_n B_1$), as has been discussed by Dimicoli and Hélène (1973), the two binding sites shall be independent and equivalent, *i.e.* they shall have the same equilibrium constant K_c' and the same complex shift $\Delta_c{}^B$. Then, it follows $K_c = 2\,K_c'$, if K_c is determined according to Eq. (3).

The models of Antonovsky *et al.* (1973) and of Dimicoli and Hélène (1973) represent the special cases of $\gamma = 1$ and $\gamma = 2$, respectively.

Since the selfassociation of the nucleobases has been evaluated using the model of Dimicoli and Hélène ($\gamma = 2$) (Schimmack *et al.*, to be published), in the present study the K_c-values have been calculated by using $\gamma = 2$ but a one-side attachment only. The chemical shifts Δ^B observed for B were fitted according to Eq. (2) by variation of $\Delta_c{}^B$. x has been calculated from Eq. (1). K_c has been determined from the minimum of the average deviation F^B of the fit, *i.e.* the difference between the shifts observed and calculated for B according to:

$$F^B(\delta_M{}^B, \Delta_c{}^B, K_c;\ \delta_M{}^A, \Delta_2{}^A, \gamma, K_s{}^A) := \frac{1}{N}\left[\sum_{n=1}^{N}\left(\Delta^{B^{(n)}}_{\text{observed}} - \Delta^{B^{(n)}}_{\text{calculated}}\right)^2\right]^{1/2}$$

N: number of samples.

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