Determination of Aqueous Solubility and pKa Values of Estrogens

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Abstract □ Reported estrone pKa and solubility data show wide variation. Improved experimental procedures were designed and used to obtain reproducible results. The pKa values for several estrogens and related compounds also were determined to assess the effects of structural differences on ionization. No evidence was obtained for long-range D to A ring electronic transmission affecting pKa. Significant differences in pKa values resulted only when conjugated unsaturation was added into the B ring of estrone or estradiol. The aqueous solubilities of estrone and 17α-estradiol were 0.8 and 3.9 μg/ml, respectively, at 25°.

Keyphrases □ Estrogens, various—aqueous solubilities, pKa values, effects of structural differences on ionization □ Solubility, aqueous—various estrogens, effects of structural differences on ionization □ pKa values—various estrogens, effects of structural differences on ionization □ Ionization—various estrogens, effects of structural differences □ Structure-activity relationships—various estrogens, aqueous solubilities and pKa values

Estrogen steroids undergo ionization in alkaline solution. Differences in the UV spectra of the ionized and neutral species of these compounds provide a suitable method for determining ionization constants (1). Previously, the spectrophotometric method was used to study the ionization constants of five estrogens (2). The same technique was also used in determining the pKa value differences between 17β-estradiol and six other estrogens (3). Considerable differences were claimed to exist between the ionization constants of the estrogens studied (2, 3). The results were explained in terms of long-range interaction and conjugation effects. Recent investigations (4, 5) of the ionization constants of 10 phenolic steroids also claimed that the character of the substitutions in the D ring would greatly affect the ionization constant of the C-3 hydroxyl group.

Reported observed ionization constant values for estrone ranged from 9.36 to 11.0 (2–7). This discrepancy may be due to differences in experimental conditions. In the present work, the thermodynamic ionization constants of nine phenolic steroids were studied spectrophotometrically. The ionization of 5,6,7,8-tetrahydro-2-naphthol also was investigated for comparison. A solubility method was employed to determine the ionization constant of equilenin. Aqueous solubility values of estrone, estradiol, equilin, and equilenin also were determined by either UV or GLC methods.

EXPERIMENTAL

Reagents—Estrone[1], 17α-estradiol[1], equilin[1], equilenin[1], 17α-dihydroequilin[1], 17β-dihydroequilenin[1], ethinyl estradiol[1], estriol[2], 1,3,5(10),6-estratetraen-3-ol-17-one (6-dehydroestrone)[2], and 5,6,7,8-tetrahydro-2-naphthol[3] were used with no prior treatment. Standard buffer solutions[4] of pH 9 and 10 were used for calibration of the glass electrode.

Apparatus—UV spectra were obtained with a recording spectrophotometer[5], and the pH was measured with a digital pH meter[6]. GLC analyses were performed on a gas chromatograph with flame-ionization detection[7], and the data were recorded with a recorder with a disk integrator[8].

Solubility Determination—Excess solid material was introduced into 200 ml of distilled water in a 250-ml jacketed beaker. The experiments were carried out at 25 ± 0.02° under nitrogen. Suspensions were magnetically stirred for at least 4 hr to allow the solution to reach equilibrium. Although 4 hr of such vigorous stirring was adequate to achieve equilibrium, several samples were stirred up to 24 hr. An aliquot of the suspension was filtered through a 0.2-μm filter membrane[9]. To prevent error due to possible filtration adsorption losses[10], at least 25 ml of suspension was first passed through the membrane. Additional suspension was collected for GLC analysis.

For GLC analysis, 5 ml of filtrate and a known amount of testosterone standard were mixed and evaporated to dryness under nitrogen. To the residue were added 15 ml of pyridine and 65 ml of *N,O*-bis(trimethylsilyl)trifluoroacetamide plus 1% trimethylchlorosilane[11]. The tube was immediately capped and mixed thoroughly on a vortex mixer[12]. The derivatized sample was injected onto a column packed with 2% DEGS on 100–120-mesh Gas Chrom Q. Analyses were performed under conditions described previously (8).

Equilenin was analyzed also by a UV method. Equilenin solubility was determined as a function of pH. The solution pH was adjusted with 0.1 *N* sodium hydroxide or 0.1 *N* hydrochloric acid, and the final pH was measured at the time of filtration. The filtrate pH was adjusted to 11.5 with 0.1 *N* sodium hydroxide. The solution concentration was determined from the absorbance at 245 nm with the molar absorptivity ($\epsilon = 5.22 \times 10^4$) for the ionized form.

Solutions for Spectrophotometric Determinations—While solution concentrations were higher than 2.0×10^{-5} *M* in previous investigations (2, 4, 5), relatively low concentrations, ranging from 2.4×10^{-6} to 1.5×10^{-5} *M* depending on the compound solubility, were used in this study. These concentrations gave either stable unsaturated or slightly oversaturated metastable solutions. The slightly oversaturated solutions were checked by UV to determine their turbidity. All solutions were stable without precipitation during the time of measurement.

Stock solutions of estrogens, 3.0×10^{-3} *M*, were prepared by dissolving weighed amounts in 100 ml of pure ethanol. Aliquots of 0.12–0.5 ml were then diluted to 100 ml with distilled water. During the dilution process, the stock solution was added dropwise with stirring to prevent localized precipitation. The solution pH was adjusted with 0.1 *N* hydrochloric acid or 0.1 *N* sodium hydroxide. The UV spectrum of the solution was recorded immediately after a stable pH reading was obtained. Spectrophotometric determinations were carried out at room temperature, 23 ± 2°.

Experimental pKa—Three distinct absorption spectra were observed for the compounds. Figure 1 shows the UV spectra of 17α-estradiol at different pH conditions. In acid solution, the molar absorptivity at the 278-nm absorption peak was 1.81×10^3 cm^{-1} *M*$^{-1}$. Two absorption peaks at 238 ($\epsilon = 9.07 \times 10^3$ cm^{-1} *M*$^{-1}$) and 296 (2.67×10^3) nm were found in alkaline solution. Other compounds with similar absorption spectra included estrone, estriol, equilin, 17α-dihydroequilin, ethinyl estradiol, and 5,6,7,8-tetrahydro-2-naphthol; they had corresponding molar absorptivities in acid solution at 278 nm of 1.85×10^3, 1.84×10^3, 1.81×10^3, 1.93×10^3, 1.89×10^3, and 2.00×10^3 cm^{-1} *M*$^{-1}$, respectively.

In alkaline solution, the molar absorptivities at the two absorption peaks were 9.26×10^3 and 2.76×10^3 cm^{-1} *M*$^{-1}$ for estrone, 9.20×10^3

[1] Ayerst Research Laboratories, Montreal, Quebec, Canada.
[2] Searle Chemical Inc.
[3] Aldrich Chemical Co.
[4] Anachemia Chemicals.
[5] Cary model 14.
[6] Corning model 110.
[7] Bendix model 2500.
[8] Honeywell model 19.
[9] Nuclepore.
[10] A. Hurwitz and S. Liu, to be published.
[11] Regisil, RC2, Regis Chemical Co.
[12] Lab-line, Melrose Park, Ill.

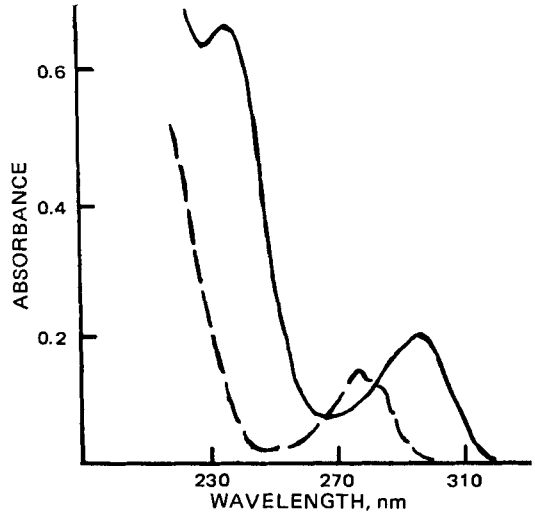

Figure 1—*UV spectrum of 17α-estradiol at a concentration of 1.50 × 10^{-5} M and a cell length of 5 cm. Key: ——, pH 12.3; and - - -, pH 3.6.*

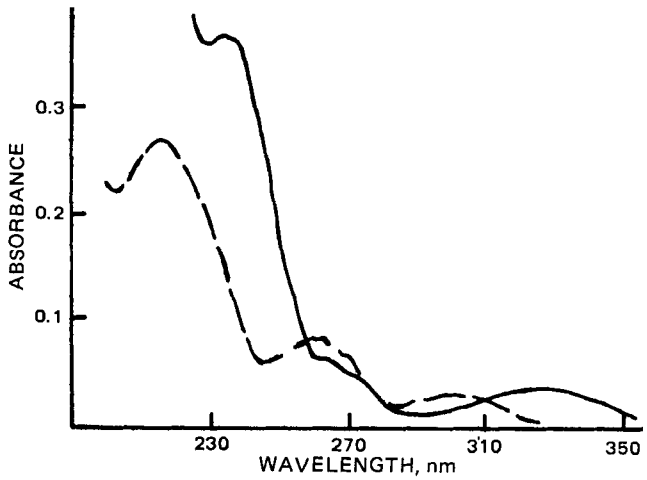

Figure 2—*UV spectrum of 6-dehydroestrone at a concentration of 2.40 × 10^{-6} M and a cell length of 5 cm. Key: ——, pH 12.0; and - - -, pH 3.0.*

and 2.73 × 10^3 cm^{-1} M^{-1} for estriol, 8.93 × 10^3 and 2.56 × 10^3 cm^{-1} M^{-1} for equilin, 8.80 × 10^3 and 2.65 × 10^3 cm^{-1} M^{-1} for 17α-dihydroequilin, 9.00 × 10^3 and 2.72 × 10^3 cm^{-1} M^{-1} for ethinyl estradiol, and 7.33 × 10^3 and 2.75 × 10^3 cm^{-1} M^{-1} for 5,6,7,8-tetrahydro-2-naphthol. Figures 2 and 3 show the UV spectrum of 1,3,5(10),6-estratetraen-3-ol-17-one and equilenin, respectively. From these figures, the influence on the UV spectra of the addition of one or two conjugated double bonds in the B ring can be seen. In contrast, equilin, having a nonconjugated double bond in the B ring, gave the same absorption spectrum as estrone.

The absorption maxima for 1,3,5(10),6-estratetraen-3-ol-17-one was at 235 (ε = 3.08 × 10^4 cm^{-1} M^{-1}) and 325 (2.58 × 10^3) nm in alkaline solution and at 215 (ε = 2.29 × 10^4 cm^{-1} M^{-1}), 261 (7.17 × 10^3), 271 (5.83 × 10^3), and 331 (2.50 × 10^3) nm in acid solution. Equilenin had maxima at 242 (ε = 5.22 × 10^4 cm^{-1} M^{-1}), 274 (6.80 × 10^3), 285 (7.47 × 10^3), 295 (4.53 × 10^3), and 355 (3.13 × 10^3) nm for the ionized form and at 228 (ε = 5.47 × 10^4 cm^{-1} M^{-1}), 269 (4.53 × 10^3), 280 (5.33 × 10^3), 292 (4.00 × 10^3), and 338 (2.47 × 10^3) nm for the neutral species.

Wavelengths selected for the pKa determinations were 240, 248, 295, and 300 nm for estrone, 17α-estradiol, estriol, ethinyl estradiol, 5,6,7,8-tetrahydro-2-naphthol, equilin, and 17α-dihydroequilin; 235 and 248 nm for 1,3,5(10),6-estratetraen-3-ol-17-one; 230, 240, and 250 nm for equilenin; and 355 and 370 nm for 17β-dihydroequilenin. All experiments were carried out using 5-cm cells, except for equilenin and 5,6,7,8-tetrahydro-2-naphthol where 2-cm cells were used.

RESULTS AND DISCUSSION

Solubility Determinations—Several studies reported the solubilities of estrone and estradiol in organic solvents (9) and aqueous solutions containing amino acids (10) or surfactants (11). However, few solubility values for estrogens in pure water are available. It was important to determine estrogen aqueous solubilities first in order to choose suitable concentration ranges for the spectrophotometric pKa determination. The improper choice of concentration may result in serious interference due to precipitation. A solubility value of 1.17 × 10^{-4} *M* was reported (12) for estrone in pure water. However, precipitation resulted when a concentrated estrone–ethanol solution was diluted in pure water to 1.5 × 10^{-5} *M*. This finding clearly indicated that the solubility of estrone in pure water is much less than 1.17 × 10^{-4} *M*.

Solubility determinations were made for estrone, 17α-estradiol, equilin, equilenin, 17α-dihydroequilin, 17α-dihydroequilenin, estriol, and ethinyl estradiol. No detectable difference in solubility values was observed for samples stirred for 4 or 24 hr. Satisfactory agreement between the UV and GLC results was found for equilenin. The experimental variation was within ±5%. Solubility values for estrone and 17α-estradiol determined by UV were about 20% to more than 50% higher than values obtained by GLC. This result indicated that these two compounds contained trace impurities of either high absorbance or relatively high solubility. Results of the GLC determinations of solubilities are shown in Table I.

The aqueous solubility observed for 17α-estradiol, 1.43 × 10^{-5} *M*, was in agreement with a literature value (1.40 × 10^{-5} *M*) obtained in 0.02 *M*

Table I—Solubility Values of Some Estrogens

Compound	Solvent	Solubility μg/ml	Solubility *M*
Estrone	Water	0.8	2.96 × 10^{-6}
17α-Estradiol	Water	3.9	1.43 × 10^{-5}
Ethinyl estradiol	Water	9.7	3.28 × 10^{-5}
Estriol	Water	3.2	1.11 × 10^{-5}
Equilin	Water	1.4	5.22 × 10^{-6}
Equilenin	pH 7.20 buffer	1.5	5.64 × 10^{-6}
	pH 8.30 buffer	1.6	6.10 × 10^{-6}
	pH 9.63 buffer	2.9	1.11 × 10^{-5}
	pH 9.85 buffer	3.6	1.37 × 10^{-5}
	pH 9.98 buffer	4.6	1.72 × 10^{-5}
	pH 10.2 buffer	6.5	2.46 × 10^{-5}
17α-Dihydroequilin	Water	10.7	3.96 × 10^{-5}
17α-Dihydroequilenin	Water	6.1	2.28 × 10^{-5}

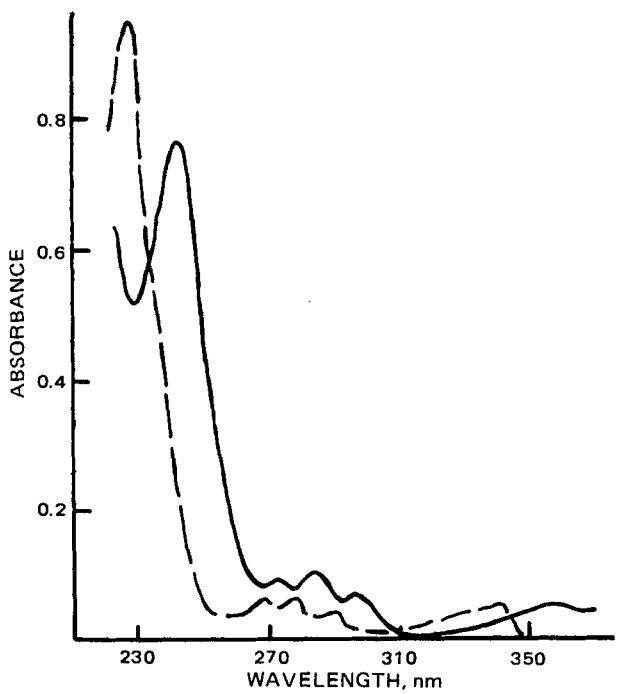

Figure 3—*UV spectrum of equilenin at a concentration of 7.5 × 10^{-6} M and a cell length of 2 cm. Key: ——, pH 12.0; and - - -, pH 3.0.*

Table II—Spectrophotometric Determination of pKa of 17α-Estradiol[a]

pH	240 nm Absorbance	240 nm pKa	248 nm Absorbance	248 nm pKa	295 nm Absorbance	295 nm pKa	300 nm Absorbance	300 nm pKa
12.3	0.670	—	0.423	—	0.200	—	0.192	—
3.6	0.047	—	0.015	—	0.007	—	0.0	—
10.37	0.342	10.42	0.218	10.38	0.096	10.44	0.089	10.43
10.51	0.360	10.51	0.227	10.48	0.102	10.52	0.098	10.49
10.38	0.326	10.47	0.200	10.46	0.095	10.46	0.090	10.44
10.65	0.421	10.47	0.265	10.45	0.120	10.50	0.115	10.47
10.30	0.298	10.47	0.180	10.47	0.086	10.46	0.080	10.45
				pKa = 10.46 ± 0.03				

[a] Cell length = 5 cm; concentration = 1.50×10^{-5} *M*.

sodium chloride solution (10). Experimental results show that estradiol is more soluble than estrone in pure water. Other investigations also revealed that estradiol has higher solubility in aqueous solutions containing surfactant materials (11). From the estrogen compounds examined, it would be expected that corresponding compounds would show a similar range of solubilities.

Spectrophotometric Determination of pKa Values—The thermodynamic ionization constant is expressed by $HA \rightleftharpoons H^+ + A^-$ and:

$$K_a = \frac{A_{H^+}[A^-]f_1}{[HA]} \quad \text{(Eq. 1)}$$

where A_{H^+} is the activity of hydrogen ion, f_1 is the activity coefficient of univalent ion, and $[A^-]$ and $[HA]$ represent the concentration of ionic and neutral species of the estrogen, respectively. At the low concentrations used, the activity coefficient, f_1, should approach 1. The following equation was employed to determine the ionization constant:

$$pKa = pH - \log\frac{[A^-]}{[HA]} = pH - \log\frac{A - A_a}{A_b - A} \quad \text{(Eq. 2)}$$

where A is the absorbance of a solution at a specific pH. The corresponding absorbances of the same concentration solution in alkaline and acid solution are A_b and A_a. The advantage of calculation with Eq. 2 and comparison of solutions at the same concentration was that the possible error due to the dilution process could be reduced. The present study was carried out in very dilute solution without salt or buffer reagents to eliminate f_1 in Eq. 1.

In previous studies, usually only one wavelength was used experimentally. However, more than one wavelength was used in the present study. Typical results for the pKa determination of 17α-estradiol are shown in Table II. Absorbance at 240 nm was about three times higher than that at 295 nm. Excellent agreement was obtained between the pKa values determined at different wavelengths. The reproducibility of these results illustrated the reliability of the present procedure. The pKa of 17α-estradiol obtained was 10.46 ± 0.03 *SD*.

A summary of the pKa values of the compounds studied is given in Table III. 17α-Estradiol, estriol, and ethinyl estradiol showed pKa values of 10.38–10.46, which were slightly higher than 10.34 observed for estrone. The difference in the pKa value between estradiol and estrone was 0.12, in good agreement with the value of 0.10 reported previously (3). The thermodynamic pKa values of estrone and 17α-estradiol observed in the present study were quite close to the apparent pKa of 10.26 for estrone and of 10.30 for estradiol reported by Egorova *et al.* (4, 5). Since these latter studies were done in buffered solutions, the slightly lower apparent pKa value reported may be due to neglected correction of the activity coefficient.

Kirdani and Burgett (2) used a spectral technique to study the ionization of five estrogens and reported a pKa value 10.91 for estrone. However, this high value resulted from certain errors in their calculations. Recalculation of their data gave a value of 10.10, close to their reported values of 10.08 for 17β-estradiol and 10.10 for 17α-estradiol.

Other reported pKa values include 11.0 for estrone determined by a back-titration technique (6) and 9.36 for estrone and 9.11 for estriol in methanol solution (7) determined by a conductiometric titration method. The great discrepancy in reported values may result from different experimental conditions. Since the present experiments were made at relatively low concentrations, possible interference due to precipitation can be eliminated. In contrast, most of the previous studies were made at quite high supersaturated conditions. For example, Egorova *et al.* (4, 5) and Kirdani and Burgett (2) employed concentrations from 3.0×10^{-5} to 8.8×10^{-5} *M*. As mentioned before, the metastable supersaturation limit of estrone in pure water is less than 1.5×10^{-5} *M*. Therefore, it would be quite difficult to prepare clear solutions, free from precipitation, at such a high concentration. The unavoidable colloidal precipitation that would result would cause serious error in the pKa determination. Furthermore, only one wavelength, 300 nm, was used (2) in the determination of the pKa values of estrone and estradiol. Due to the low absorbance at this wavelength, any interference would cause a large error in the measurements. The advantages of using stable, low concentration solutions and different wavelengths were illustrated by the good agreement obtained for pKa value determinations.

Table III—pKa Values of Several Estrogens and Related Compounds

Compound	Number of Determinations	Concentration, *M*	pKa	Literature pKa
Estrone	5	7.5×10^{-6}	10.34 ± 0.05	10.91 (2), 10.26 (4, 5)
	2	5.4×10^{-6}	—	—
17α-Estradiol	5	1.5×10^{-5}	10.46 ± 0.03	10.10 (2), 10.30 (4, 5)
Estriol	3	1.5×10^{-5}	10.38 ± 0.02	—
	2	9.0×10^{-6}	—	—
Ethinyl estradiol	6	1.5×10^{-5}	10.40 ± 0.01	—
Equilin	3	1.5×10^{-5}	10.26 ± 0.04	—
	4	9.0×10^{-6}	—	—
17α-Dihydroequilin	2	1.5×10^{-5}	10.29 ± 0.02	—
	3	9.0×10^{-6}	—	—
6-Dehydroestrone	4	2.4×10^{-6}	10.17 ± 0.04	—
Equilenin	4	7.5×10^{-6}	9.75 ± 0.06	—
17β-Dihydroequilenin[a]	5	—	9.77 ± 0.04	—
Phenol	—	—	—	10.00 (1)
m-Cresol	—	—	—	10.09 (1)
p-Cresol	—	—	—	10.26 (1)
5,6,7,8-Tetrahydro-2-naphthol	6	1.5×10^{-5}	10.51 ± 0.02	10.32 (1) (20°)
1-Naphthol	—	—	—	9.30 (1) (20°)
2-Naphthol	—	—	—	9.63 (1) (20°)

[a] The material used also contained 17β-dihydroequilin. Determinations were made at 370 nm to prevent interference.

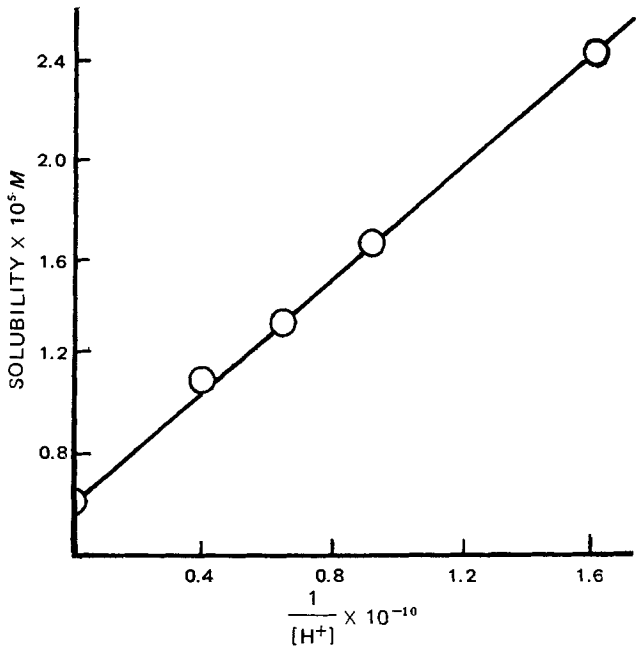

Figure 4—*Plot of solubility* versus *the reciprocal of the hydrogen-ion concentration for equilenin.*

In previous studies (2–5), small pKa value differences between estrone and estradiol were explained in terms of long-range D to A ring transmission effects. While conformational or steric transmission effects have important consequences in some reaction systems, it is not likely that electronic transmission affecting pKa occurs for estrone and estradiol. The experimental data obtained show small differences in pKa values. However, these differences apparently fall within anticipated experimental reproducibility ranges. No significant effect on pKa values occurred when the C-17 substituent was varied between carbonyl and hydroxyl for three pairs of compounds. Values for estrone, 17α-estradiol, ethinyl estradiol, and estriol ranged from 10.34 to 10.46. Where an unconjugated double bond was present in the B ring, as in equilin and 17α-dihydroequilin, pKa values were 10.26 and 10.29, respectively. When a single double bond in the B ring was in conjugation with the A ring, as in 1,3,5(10),6-estratetraen-3-ol-17-one, the pKa value observed was 10.17. For fully unsaturated B ring compounds such as equilenin and 17β-dihydroequilenin, pKa values were 9.75 and 9.77, respectively.

The acidity of the estrogens was increased by introduction of one or more double bonds in the B ring. The pKa values of estrone and related derivatives followed the order estrone > equilin > 1,3,5(10),6-estratetraen-3-ol-17-one > equilenin. The conjugated double bond in 1,3,5(10),6-estratetraen-3-ol-17-one had a greater effect in increasing acidity than the nonconjugated double bond in the B ring of equilin. Among the compounds examined, the fully conjugated B ring of equilenin produced the most profound effect in reduced pKa value. The pKa of equilenin was 0.59 unit lower than that of estrone. Similar inductive effects were found among estradiol, 17α-dihydroequilin, and 17β-dihydroequilenin.

Literature pKa values of phenol and some simple derivatives are listed in Table III for comparison. Comparison of the pKa values of 5,6,7,8-tetrahydro-2-naphthol and phenol shows that the attachment of a saturated B ring at the *meta*- and *para*-positions caused an increase in pKa of 0.5 unit. By contrast, a fully unsaturated naphthol such as 1-naphthol or 2-naphthol showed an increased acidity greater than that of phenol. The results with the more complicated estrogens were consistent with these general observations.

Solubility Method Determination of Equilenin pKa—The phenolic steroids studied are quite insoluble weak acids. Solubility changes as a function of pH provide a useful method for determining the ionization constants of these compounds. Equilenin was chosen for the comparison of pKa determination methods. Solubility values of equilenin at different pH values are listed in Table I. It was expected that a sharp increase in solubility would occur at pH values near or greater than the pKa value. Solubility values, S, can be expressed for the present experimental conditions by:

$$S = [HA] + [A^-] = [HA] + \frac{K_a[HA]}{[H^+]} \qquad \text{(Eq. 3)}$$

where [HA] is the intrinsic solubility of the neutral or undissociated species of equilenin. A linear relationship was found between S and $1/[H^+]$, as shown in Fig. 4. A value of [HA] can be obtained either from the intercept of this plot or by direct measurement in acid solution. The value for [HA] obtained from direct measurement was 5.71×10^{-6} M, which was close to that obtained from the intercept, 6.10×10^{-6} M. Linear regression was employed to determine the slope of the plot. The association constant was calculated from the equation: slope = $K_a \times [HA] = 1.13 \times 10^{-15}$. The pKa thus obtained was 9.72, in excellent agreement with the value obtained by spectrophotometric determination.

This solubility technique could also be applied in the determination of the ionization constants of other phenolic steroids. However, if a spectrophotometric method is employed for analysis, special precautions must be taken to avoid possible interference by impurities.

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ACKNOWLEDGMENTS AND ADDRESSES

Received March 15, 1976, from the *Pharmaceutical Research and Development Division, Ayerst Laboratories, Inc., Rouses Point, NY 12979.*

Accepted for publication June 17, 1976.

The authors thank Dr. R. Johnson for cooperation and assistance in obtaining the estrogen samples and are grateful for the technical assistance of Mr. R. Haring in performing GLC analysis.

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