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Simultaneous Solubilization of Steroid Hormones III: Thermodynamic Evaluation

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Received December 5, 1978, from the *Department of Biochemistry and Pharmacy, Åbo Akademi, Porthansgatan 3, 20500 Åbo 50, Finland.* Accepted for publication July 11, 1979.

Abstract □ The temperature effect on the solubilization of some androgens, estrogens, and C_{21}-steroids in aqueous polysorbate 40 and in tetradecyltrimethylammonium bromide was studied. Dialysis studies showed a linear relationship between micellar and nonmicellar steroids, which indicates that solubilization is governed by a distribution coefficient. With known water solubilities and solubilization capacities for the steroids at different temperatures, the changes of free energy (ΔG_s°), enthalpy (ΔH_s°), and entropy (ΔS_s°) for solubilization were calculated. All steroids studied had negative ΔH_s° values in polysorbate 40, except progesterone and ethisterone. The ΔS_s° values were positive for all of the actual steroids except for estradiol in both association colloids and for 17α-hydroxyprogesterone in polysorbate 40. The highest values were obtained for progesterone and testosterone. The steroids showed lower ΔS_s° values when they were solubilized simultaneously than when they were solubilized separately. No clearcut correlation between the entropy change of solubilization and the simultaneous solubilization behavior could be derived. Obviously, the solubilization mechanism also must be considered. The thermodynamic solubilization parameters are discussed, and the need for temperature-solubilization studies is stressed.

Keyphrases □ Steroid hormones—solubilization, thermodynamics, temperature effect, micellar structure, androgens, estrogens, C_{21}-steroids, in polysorbate 40 and in tetradecyltrimethylammonium bromide □ Solubilization—steroid hormones in polysorbate 40 and in tetradecyltrimethylammonium bromide, thermodynamics □ Thermodynamics—steroid hormone solubilization in polysorbate 40 and in tetradecyltrimethylammonium bromide

Two previous reports from this laboratory dealt with the simultaneous solubilization of estrogens, C_{21}-steroids, and androgens in aqueous solutions of association colloids (1, 2). Estradiol is solubilized independently of the C_{21}-steroids and testosterone, while the solubilization of ethinyl estradiol with progesterone and with testosterone is dependent. A plausible mechanism for simultaneous solubilization was discussed (2). However, mere solubilization capacities at one temperature are not a good basis for thermodynamic discussion of the solubilization mechanism. In this study, the temperature effect on solubilization was investigated to elucidate the contributions of enthalpy and entropy to the free energy of solubilization.

The thermodynamic parameters controlling micellization have been studied and discussed (3–5), but the corresponding parameters for solubilization have been comparatively neglected. One difficult question is the choice of a model for thermodynamic parameter calculations. Humphreys and Rhodes (6) found that the micellar pseudophase model seemed applicable because solubilization was governed by a form of the distribution law. Plots for the determination of enthalpic and entropic values were complex.

More straightforward results were obtained by Simons and Rhodes (7) with a linear relationship between the free energy of solubilization and temperature, despite much scatter due to experimental difficulties. Their results favor the pseudophase model.

EXPERIMENTAL

Materials—The purification methods and purity tests for the association colloids and steroid hormones[1] were described previously (1), except that the steroid purity was checked by silica gel TLC. The labeled steroids[2], ^{3}H-estradiol, ^{3}H-progesterone, and ^{3}H-testosterone, had a radiochemical purity of 98% by TLC, and they were used without further purification. The association colloids were tetradecyltrimethylammonium bromide[3] and polysorbate 40[4].

Solubilization—Solubility studies were carried out as described previously (1), but samples were filtered through a 0.45-μm filter membrane[5] before steroid quantitation. The UV absorbance was used to calculate the amount of unlabeled steroid solubilized; liquid scintillation counting, with a toluene-based scintillation cocktail, was used for the labeled steroids.

The equilibration temperatures were controlled to ±0.2°. The equilibrium dialysis was performed using dialysis tubing[6]. Complete equilibration of the solutions was ensured. All of the experiments were done at least twice.

RESULTS AND DISCUSSION

Dialysis studies were done with the three ^{3}H-labeled steroids: estradiol, progesterone, and testosterone. Figure 1 shows typical results. The linearity of such plots confirms that the relationship between the micellar and nonmicellar steroid concentrations is linear in both saturated and nonsaturated systems. This linearity indicates that solubilization in these systems is governed by a distribution coefficient.

The solubilization capacities at different temperatures between 293 and 323 °K of the two surfactants (tetradecyltrimethylammonium bromide and polysorbate 40) for the sex steroids (estradiol, ethinyl estradiol, progestone, 17α-hydroxyprogesterone, testosterone, and ethisterone) were calculated from saturation solubilization experiments (Table I).

The temperature effect on simultaneous solubilization was studied with the following combinations: ethinyl estradiol–progesterone, ethinyl estradiol–17α-hydroxyprogesterone, and estradiol–testosterone, all in tetradecyltrimethylammonium bromide; and ethinyl estradiol–proges-

[1] Fluka AG. Schweiz.
[2] The Radiochemical Centre, England.
[3] K & K Laboratories.
[4] Atlas Chemical Industries.
[5] Millipore Corp.
[6] Med Cell International Ltd., England.

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Table I—Solubilization Capacities of Surfactants for Hormonal Steroids as a Function of Temperature

Surfactant	Steroid	Moles of Steroid per Mole of Surfactant				
		293 °K	300.5 °K	308 °K	315.5 °K	323 °K
Polysorbate 40	Estradiol	0.013	0.016	0.019	0.022	0.026
	Ethinyl estradiol	0.18	0.23	0.27	0.32	0.37
	Testosterone	0.027	0.039	0.052	0.065	0.076
	Ethisterone	0.0007	0.0009	0.0012	0.0016	0.0018
	Progesterone	0.037	0.049	0.063	0.073	0.084
	17α-Hydroxyprogesterone	0.0072	0.0079	0.0085	0.0091	0.0091
Tetradecyltrimethylammonium bromide	Estradiol	0.068	0.080	0.092	0.105	0.118
	Ethinyl estradiol	0.27	0.34	0.43	0.51	0.57
	Testosterone	0.13	0.19	0.25	0.29	0.35
	Ethisterone	0.0046	0.0055	0.0066	0.0074	0.0083
	Progesterone	0.16	0.15	0.16	0.16	0.16
	17α-Hydroxyprogesterone	0.043	0.060	0.082	0.098	0.114

Table II—Solubilization Capacities of Surfactants for Simultaneous Solubilization of Hormonal Steroids as a Function of Temperature

Surfactant	Steroid Pair	Moles of Steroid per Mole of Surfactant				
		293 °K	300.5 °K	308 °K	315.5 °K	323 °K
Polysorbate 40	Ethinyl estradiol +	0.034	0.038	0.042	0.047	0.053
	progesterone	0.037	0.038	0.037	0.036	0.036
	Ethinyl estradiol +	0.063	0.070	0.075	0.082	0.087
	testosterone	0.027	0.031	0.036	0.041	0.045
	Estradiol +	0.013	0.014	0.016	0.018	0.020
	progesterone	0.037	0.041	0.046	0.051	0.056
Tetradecyltrimethylammonium bromide	Ethinyl estradiol +	0.037	0.043	0.049	0.055	0.060
	progesterone	0.16	0.20	0.23	0.27	0.30
	Ethinyl estradiol +	0.19	0.23	0.27	0.31	0.36
	17α-hydroxyprogesterone	0.0054	0.0072	0.0090	0.104	0.0120
	Estradiol +	0.013	0.016	0.018	0.021	0.023
	testosterone	0.027	0.036	0.047	0.058	0.068

terone, ethinyl estradiol–testosterone, and estradiol–progesterone, all in polysorbate 40 (Table II).

The linear relationship between the surfactant concentration and the amount of solubilized steroid (1, 2) as well as the linearity of the dialysis plots (Fig. 1) indicates that the solubilization is governed by a form of the distribution law. The equilibrium constant, K_s, between the micellar (mole fraction of the solute in the micelle, C_s^m) and the nonmicellar (aqueous solubility of the steroid in moles per liter, C_s^{aq}) steroid can be defined by (2, 6):

$$K_s = C_s^m/C_s^{aq} \qquad \text{(Eq. 1)}$$

Starting with the thermodynamic equilibrium constant for the solubilization, one can calculate the free energy change of solubilization, ΔG_s°:

$$\Delta G_s^\circ = -RT \ln K_s \qquad \text{(Eq. 2)}$$

Figures 2*a* and 2*b* show the variation of the free energy change of solubilization with temperature for steroid hormones in the two association colloids. The ΔG_s° values are calculated starting with the water solubility of the steroids at different temperatures (8) and the solubilization capacities at the corresponding temperatures. Most curves show a fairly linear relationship between ΔG_s° and temperature, which also was found for testosterone solubilization by *n*-alkyl polyoxyethylenes (7). The curves for estradiol and ethinyl estradiol in both surfactants show the greatest deviations from linearity. The most likely explanation for such curvature would be the variation of micellar structure with temperature (6). Estradiol and ethinyl estradiol also had the most negative ΔG_s° values, especially at lower temperatures. These facts may indicate a different solubilization mechanism for these steroids compared to the others.

The ΔG_s° values of testosterone and ethisterone differed only slightly. This finding is remarkable if one considers the much higher solubilization capacity for testosterone compared to ethisterone (2). Progesterone had more negative ΔG_s° values than 17α-hydroxyprogesterone, especially in polysorbate 40.

Estradiol and ethinyl estradiol also gave deviations from linearity in ΔG_s° *versus* T plots (Figs. 3*a* and 3*b*) when solubilized simultaneously with other steroids. Ethinyl estradiol gave more positive ΔG_s° values in combinations with progesterone, testosterone, and 17α-hydroxyprogesterone than when solubilized alone. 17α-Hydroxyprogesterone gave more positive values when solubilized with ethinyl estradiol. There was, however, no clearcut correlation between the ΔG_s° values of the steroids and their behaviors at simultaneous solubilization.

With the assumption that the standard molar enthalpy change is not temperature dependent in the range studied, Eq. 3 can be used:

$$\frac{d \ln K_s}{dT} = \frac{\Delta H_s^\circ}{RT^2} \qquad \text{(Eq. 3)}$$

The standard solubilization enthalpy change, ΔH_s°, may be obtained from the plot of $\ln K_s$ *versus* $1/T$. Such plots are found in Figs. 4 and 5 for steroids solubilized separately and simultaneously. The ΔH_s° values are summarized in Tables III and IV.

Testosterone had low negative ΔH_s° values when solubilized separately in both polysorbate 40 and tetradecyltrimethylammonium bromide. Testosterone had somewhat higher negative values when solubilized simultaneously with ethinyl estradiol in polysorbate 40 and with estradiol in tetradecyltrimethylammonium bromide. Ethinyl estradiol had moderately negative values when solubilized either separately or simultaneously and in both association colloids. Estradiol showed the largest negative values.

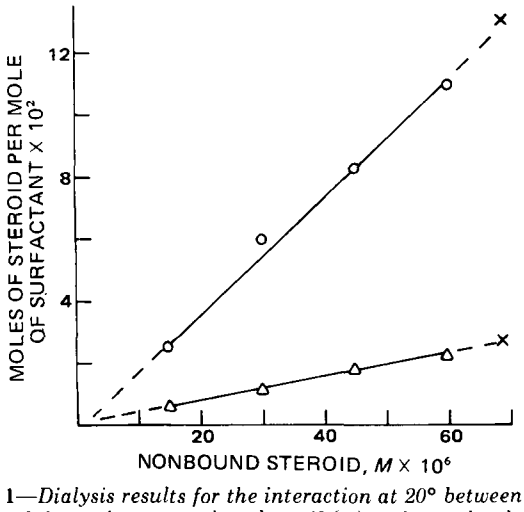

Figure 1—*Dialysis results for the interaction at 20° between testosterone and the surfactants polysorbate 40* (△) *and tetradecyltrimethylammonium bromide* (○) (× = *solubility points*).

Table III—Thermodynamic Changes [a] in Solubilization of Hormonal Steroids in Surfactants at 293°K

Surfactant	Steroid	$-\Delta G_s^\circ$, joules/mole	$-\Delta H_s^\circ$, joules/mole	ΔS_s°, joules/deg/mole
Polysorbate 40	Estradiol	18,631	28,018	−32
	Ethinyl estradiol	21,578	8,314	45
	Testosterone	14,552	1,081	46
	Ethisterone	14,816	−699	53
	Progesterone	17,174	−11,889	99
	17α-Hydroxyprogesterone	14,993	27,686	−43
Tetradecyltrimethylammonium bromide	Estradiol	22,661	32,674	−34
	Ethinyl estradiol	22,566	8,563	48
	Testosterone	20,740	2,744	61
	Ethisterone	19,344	5,570	47
	Progesterone	18,381	11,640	23
	17α-Hydroxyprogesterone	19,400	9,727	33

[a] ΔG_s°, ΔH_s°, and ΔS_s° refer to the unitary free energy, enthalpy, and entropy of solubilization, respectively.

17α-Hydroxyprogesterone had a large negative value in polysorbate 40 but had considerably lower values when alone and when together with ethinyl estradiol in tetradecyltrimethylammonium bromide. Progesterone showed the most ambivalent behavior. Solubilized separately and together with ethinyl estradiol in polysorbate 40, it gave positive ΔH_s° values. Alone in tetradecyltrimethylammonium bromide and together with estradiol and ethinyl estradiol in polysorbate 40, progesterone gave negative values.

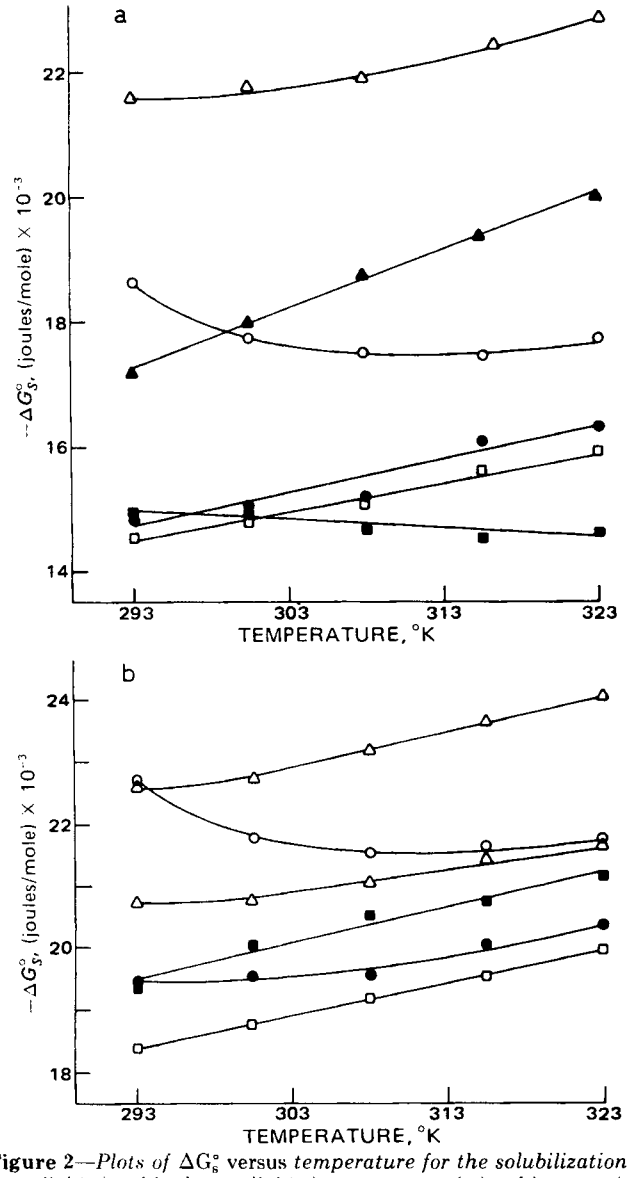

Figure 2—*Plots of* ΔG_s° versus *temperature for the solubilization of estradiol* (○), *ethinyl estradiol* (△), *testosterone* (□), *ethisterone* (●), *progesterone* (▲), *and 17α-hydroxyprogesterone* (■) *in polysorbate 40* (a) *and in tetradecyltrimethylammonium bromide* (b).

Starting with the ΔG_s° and ΔH_s° values, one can calculate the standard entropy change of solubilization, ΔS_s°:

$$\Delta G_s^\circ = \Delta H_s^\circ - T\Delta S_s^\circ \qquad \text{(Eq. 4)}$$

The ΔS_s° values for separate and simultaneous solubilization appear in Tables III and IV. The steroids showed large differences in entropy values. All of the steroids except estradiol and 17α-hydroxyprogesterone in polysorbate 40 had positive values. Progesterone in polysorbate 40 and testosterone in tetradecyltrimethylammonium bromide showed the highest values. At simultaneous solubilization, all steroids had lower entropy values than when solubilized individually, except progesterone in tetradecyltrimethylammonium bromide.

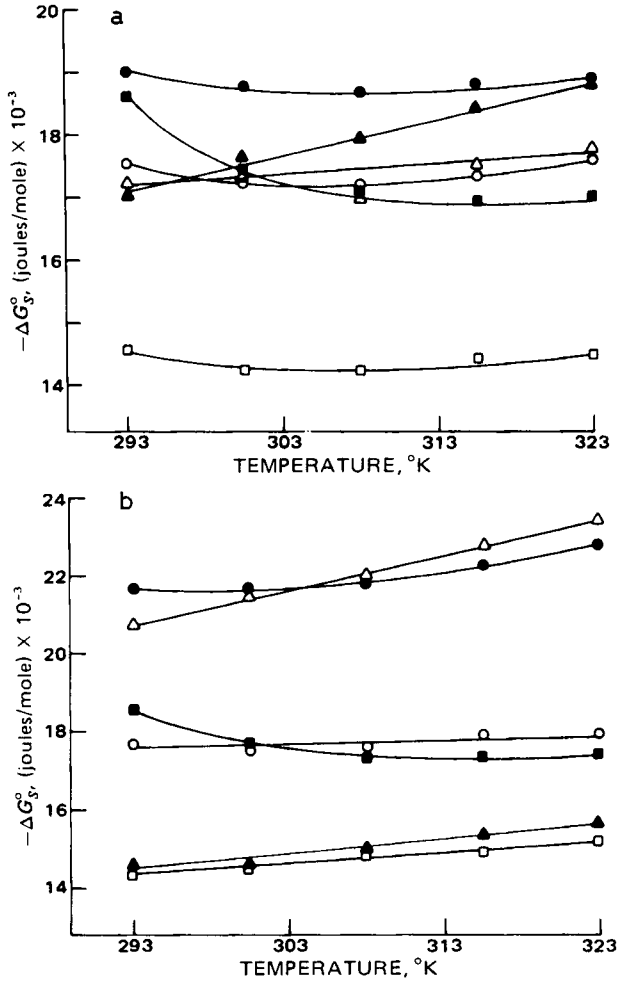

Figure 3—*Plots of* ΔG_s° versus *temperature for the simultaneous solubilization of* (a) *ethinyl estradiol* (○) *plus progesterone* (△), *ethinyl estradiol* (●) *plus testosterone* (□), *and estradiol* (■) *plus progesterone* (▲) *in polysorbate 40 and of* (b) *ethinyl estradiol* (○) *plus progesterone* (△), *ethinyl estradiol* (●) *plus 17α-hydroxyprogesterone* (□), *and estradiol* (■) *plus testosterone* (▲) *in tetradecyltrimethylammonium bromide.*

Table IV—Thermodynamic Changes[a] in Simultaneous Solubilization of Hormonal Steroids in Surfactants at 293°K

Surfactant	Steroid Pair	$-\Delta G_s^\circ$, joules/mole	$-\Delta H_s^\circ$, joules/mole	ΔS_s°, joules/deg/mole
Polysorbate 40	Ethinyl estradiol +	17,518	18,041	−2
	progesterone	17,174	11,058	21
	Ethinyl estradiol +	19,021	19,954	−3
	testosterone	14,552	15,797	−4
	Estradiol +	18,631	36,582	−61
	progesterone	17,174	−690	61
Tetradecyltrimethylammonium bromide	Ethinyl estradiol +	17,724	15,963	6
	progesterone	20,740	−5,570	90
	Ethinyl estradiol +	21,710	11,640	34
	17α-hydroxyprogesterone	14,291	6,901	25
	Estradiol +	18,631	32,591	−48
	testosterone	14,552	4,988	33

[a] ΔG_s°, ΔH_s°, and ΔS_s° refer to the unitary free energy, enthalpy, and entropy of solubilization, respectively.

A comparison of the contribution of the enthalpy and entropy changes to the free energy change of solubilization displayed large differences among the steroids. For estradiol in both surfactants, 17α-hydroxyprogesterone in polysorbate 40, and progesterone in tetradecyltrimethylammonium bromide, the enthalpy factor made the largest contribution to the negative free energy change. For the other steroids, the entropy was larger, and solubilization was thus essentially an entropy-driven process. The same situation generally applies to micelle formation from detergent molecules, which is accompanied by very small heat changes and is assumed to be controlled by a large ΔS.

The most popular explanation of the large unitary entropy change involved in the partitioning of hydrophobic molecules between aqueous and nonaqueous phases is the flickering cluster hypothesis (9). When organic compounds are placed in water, the water molecules arrange themselves around the apolar parts in flickering clusters. Stripping the water molecules from the apolar part of the solute results in large entropy and in the randomization of the water molecules. This treatment assumes that solubilization can be described as the transfer of a molecule from an aqueous to a nonaqueous phase. Application of this assumption to

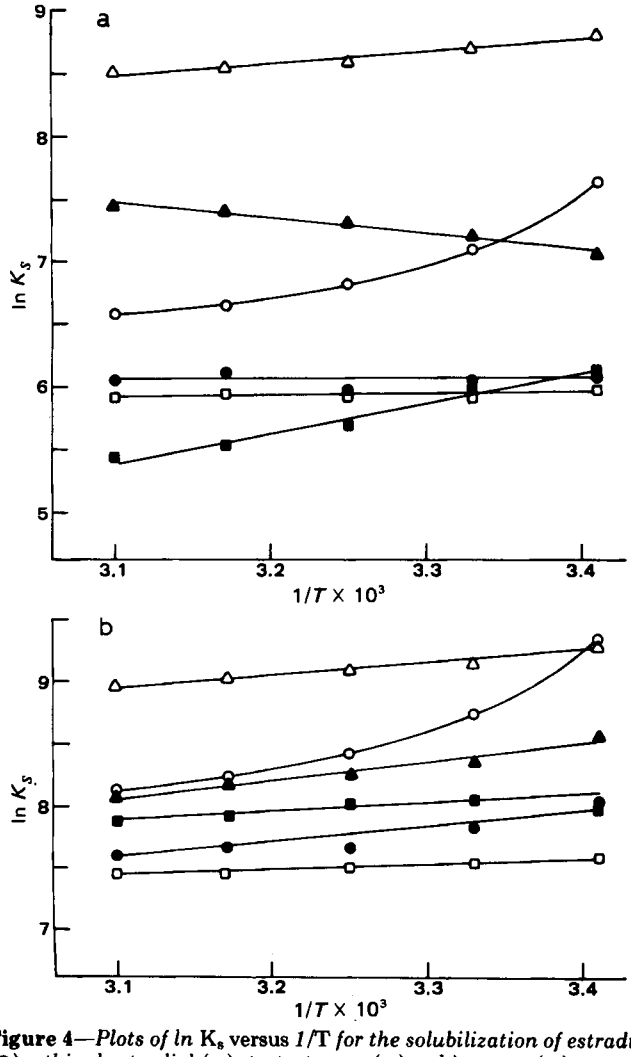

Figure 4—*Plots of ln* K_s versus *1/T for the solubilization of estradiol* (○), *ethinyl estradiol* (△), *testosterone* (□), *ethisterone* (●), *progesterone* (▲), *and 17α-hydroxyprogesterone* (■) *in polysorbate 40* (a) *and tetradecyltrimethylammonium bromide* (b).

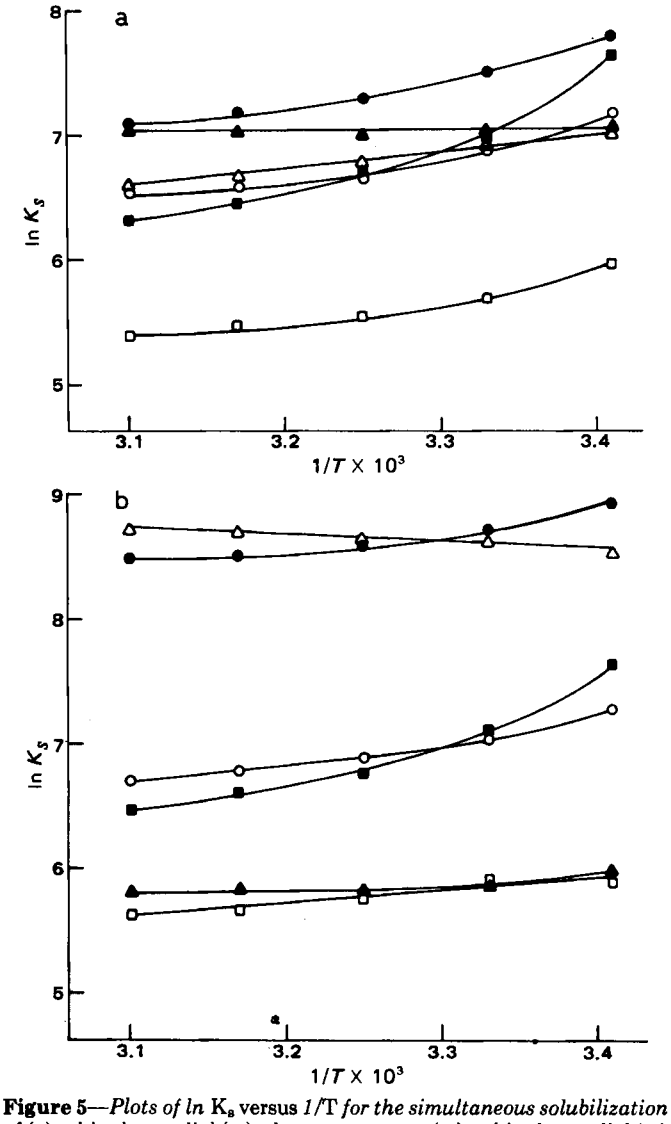

Figure 5—*Plots of ln* K_s versus *1/T for the simultaneous solubilization of* (a) *ethinyl estradiol* (○) *plus progesterone* (△), *ethinyl estradiol* (●) *plus testosterone* (□), *and estradiol* (■) *plus progesterone* (▲) *in polysorbate 40 and of* (b) *ethinyl estradiol* (○) *plus 17α-hydroxyprogesterone* (△) *and estradiol* (■) *plus testosterone* (▲) *in tetradecyltrimethylammonium bromide.*

steroid hormone solubilization is supported by the similarity between the thermodynamic parameters found in this study and those from octanol–water partitioning (8).

Although the flickering cluster hypothesis seems plausible, and the water ordering–disordering is important for the entropy change of solubilization, other factors may be significant. It was proposed (10) that the apolar solute molecule is held rigidly in a favored rotational configuration in the aqueous phase by the layer of water molecules surrounding it. In the micelle hydrocarbon core, its rotational oscillations are relatively unrestricted. This proposal is supported by the fact that there is no clearcut correlation between water solubility and ΔS_s°. Ethisterone has a much lower water solubility than testosterone [1.6 against 68.7 μmoles/liter at 20° (8)] and can be regarded as more hydrophobic. According to the flickering cluster hypothesis, ethisterone should have more structured water around it and should give a more positive ΔS_s° at the randomization of the water molecules. Thus, both the water and solute effects probably contribute to the effective ΔS_s°, but which is quantitatively the most important cannot be determined.

The contribution of the enthalpy change to the free energy change of solubilization is quantitatively minor for most steroids. Hence, there are interesting differences in their ΔH_s° values (Table III). The most notable difference is the rather large positive value of progesterone in polysorbate 40. Because similar ΔH_s° values can be expected for steroids solubilized by the same mechanism, the differences obtained indicate variations in that respect.

The simultaneous solubilization of steroids cannot be predicted by their free energies of solubilization (2). Testosterone and progesterone have less negative ΔH_s° values than ethinyl estradiol but are solubilized maximally in polysorbate 40, while the latter steroid has a lower solubility at simultaneous solubilization. These discrepancies may be explained by differences in the solubilization mechanism. If the solubilization loci of the steroids partly overlap, a steric hindrance to simultaneous solubilization will exist. When solubilized on its own in tetradecyltrimethylammonium bromide, ethinyl estradiol has a considerably larger ΔS_s° than progesterone, but the opposite is true at simultaneous solubilization (Tables III and IV).

On the contrary, progesterone and estradiol are solubilized independently of each other in polysorbate 40, and the ΔS_s° values of both decrease to about the same extent when the steroids are solubilized together. Also, the solubilizations of both testosterone and estradiol are changed at simultaneous solubilization in tetradecyltrimethylammonium bromide, and their ΔS_s° values are both lowered when the steroids are solubilized together. However, at this stage it is hard to rationalize these results in terms of the precise structure and ordering of the micelle and the solute in it.

Of course, the thermodynamic treatment in this study has limitations. Although the amount of solubilizate bound in a micellar system can be measured with accuracy, the calculation of C_s^m presents problems because it is a concentration term, and precise delineation of the micellar pseudophase boundary is difficult. Two other difficult aspects of the thermodynamic calculations regarding micellar binding are the selection of the standard state for the micellar cosolute and the incorporation of the appropriate activity corrections (11). For cosolutes with low water solubility such as steroid hormones, the C_s^{aq} term probably will approximate the activity value. However, the micellar solubilizate activity coefficients may differ significantly from unity. This deviation will introduce errors into the calculations of thermodynamic parameters derived from micellar binding equilibrium constants.

Although these limitations exist, data from studies of the temperature effect on solubilization processes can prove useful in expanding knowledge of this important branch of surface chemistry. Clearly, further studies of the effect of solutes on micelle structure are required before a theory rationalizing micellar solubilization can be formulated.

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Tetrazolium Salts in Pharmaceutical Analysis II: Direct Assay of Diethylstilbestrol and Diethylstilbestrol Dipropionate

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Received November 24, 1978, from the *Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Assiut, Assiut, Egypt.* Accepted for publication July 10, 1979.

Abstract □ A convenient spectrophotometric determination of diethylstilbestrol and diethylstilbestrol dipropionate was developed involving their interaction with triphenyltetrazolium chloride at 50° for 45 min and subsequent measurement of the formazan formed. The significance of extended conjugation within the 4,4′-stilbenediol molecule to induce the color reaction is documented. Ideal adherence of color absorption to Beer's law permitted accurate and precise determination of diethylstilbestrol and diethylstilbestrol dipropionate pure forms over the range of 2–22 μg of diethylstilbestrol/ml. Application of the tetrazolium color reaction to the analysis of diethylstilbestrol dipropionate dosage forms was achieved without prior hydrolysis or extraction.

Keyphrases □ Diethylstilbestrol and diethylstilbestrol dipropionate—analysis, triphenyltetrazolium chloride colorimetry □ Colorimetry—analysis of diethylstilbestrol and diethylstilbestrol dipropionate with triphenyltetrazolium chloride □ Tetrazolium salts—colorimetric analysis of diethylstilbestrol and diethylstilbestrol dipropionate

The usual relatively small doses of diethylstilbestrol [(*E*)-α,α′-diethyl-4,4′-stilbenediol] require especially sensitive and precise pharmaceutical analysis. The phenolic hydroxyl group reactivity of this stilbene derivative has been used to develop diverse estimation procedures based on acetylation (1, 2), nitrosation (3), polarography of the nitrosation product (4), bromination (5), and UV irradiation (6).

Of the chromogenic reagents reported for interaction with phenols, interest has focused on the utility of phosphomolybdotungstate (7), iron (8), antimony (9), and vanadium (10) salts for diethylstilbestrol colorimetry. Some

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