# STEROIDS AND STEROIDASES XVII (1). DELINEATION OF SOME FACTORS INVOLVED IN THE FORMATION AND DISAGGREGATION OF STEROID MICELLES IN AQUEOUS SOLUTIONS USING ACID-CATALYZED ISOMERIZATION OF $\Delta^5$-3-KETONES AS A CONVENIENT KINETIC METHOD FOR MONITORING THE PROCESSES.

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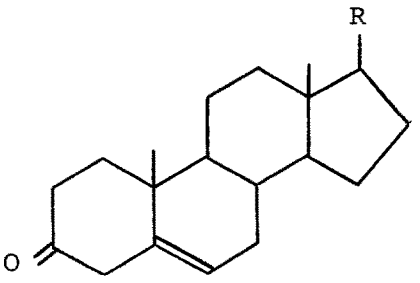
## ABSTRACT

Some of the factors involved in the formation and disaggregation in aqueous solutions of micelles of $\Delta^5$-3-ketosteroids possessing 17β-H, $CH_3$, $CH_2CH_3$, $CH(CH_3)_2$ and $C_8H_{17}$ substituents have been evaluated using both light-scattering and acid-catalyzed isomerization kinetic techniques. The latter method has been shown to be a convenient and sensitive probe for monitoring the degree of substrate aggregation and for determining the conditions under which complete solvation occurs. Studies of the relative effectiveness of organic solvents, particularly of homologous alcohols, in causing micelle break-up have indicated that disruption of the steroid aggregates occurs by solvation of the hydrophobic skeleton by the organic solvent. The steroid micelles appear to resemble those of nonionic surfactants or lipids and a general structure for the aggregates in aqueous solution is proposed in which overlap of the hydrophobic steroid framework is maximized.

## INTRODUCTION

Our interest in the aggregation of steroids in aqueous solutions was prompted by the indication that formation of micelles by substrates possessing non-polar C-17 groups was an important factor in determining the apparent specificity of the $\Delta^5 \rightarrow \Delta^4$-3-ketosteroidisomerase of Pseudomonas testosteroni (2). Accordingly, in order to determine the influence of the overall hydrophobic - hydrophilic balance of the substrate in this regard, and of the C-17 substituent in particular, a systematic study of the series of $\Delta^5$-3-ketosteroids 1a-e in which the C-17 groups become increasingly hydrophobic, was undertaken using an acid-catalyzed isomerization kinetic technique to monitor their aggregation tendencies. The previous studies (2) on the kinetics of acid-catalyzed isomerizations of aqueous solutions of the relatively hydrophobic $\Delta^5$-3-ketones 1a and 1e had shown that the presence of aggregates was reflected in a multiple-order dependence on the steroid concentration. Progressive disruption of the micelles was achieved by increasing the methanol content of the solutions and when the methanol concentration reached the minimum level required to achieve complete solvation of the substrate, kinetic plots of the pseudo first-order type were observed. The current investigations have extended the scope of this method and the data obtained confirm it to be a very convenient one for studying the

aggregation properties of $\Delta^5$-3-ketosteroids. To our knowledge it is the only application of a kinetic procedure for detecting the onset of micelle formation yet reported.

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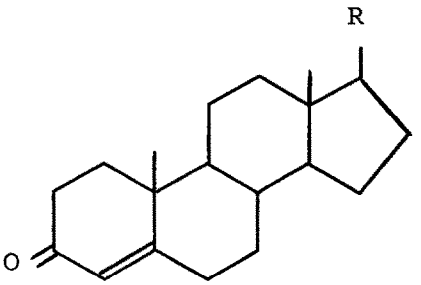


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a, R = H

b, R = $CH_3$

c, R = $CH_2CH_3$

d, R = $CH(CH_3)_2$

e, R = $C_8H_{17}$

## MATERIALS AND METHODS

The steroids 1a-e and 2a-e were obtained or prepared as described previously (2,3). KCl-HCl buffer solutions were made up according to the procedure of Gomori (4). All solvents used were ACS grade (Fisher) and were purified (5) before use. Dust-free, distilled water was used for all aqueous solutions. Stock solutions of the steroids were made up in the particular organic solvent being evaluated.

### General Procedure for Acid-Catalyzed Isomerization of the $\Delta^5$-3-Ketones 1a-e

All isomerizations were performed as described previously (2) at 25°C in 1.0 cm pathlength quartz cuvettes using a Beckmann DU spectrophotometer to follow the rate of increase of absorbance (in O.D. units) at the wavelength of maximum absorption of the $\Delta^4$-3-ketone product (242-244 nm). The 3 ml reaction volumes were composed of 0.2M KCl-HCl buffer pH 1.0 (6) ([2.95-x]ml), organic co-solvent (x ml), and 1.5 mM $\Delta^5$-3-ketosteroid stock solution (0.05 ml). The amount of organic co-solvent was gradually increased until the isomerization followed pseudo first-order kinetics.

All runs were carried out at least in duplicate and were reproducible to $\lesssim \pm 3\%$. The data were treated in the usual first-order manner by plotting $\log_{10}\Delta OD$ vs t, where $\Delta OD$ represents the difference between the optical density reading at infinite time (at least 6 half-lives) and that at any time t.

Light Scattering Measurements on Aqueous Solutions of the $\Delta^4$-3-Ketones 2a-e

The light scattering experiments were carried out on a Brice-Phoenix Universal 1000 Series, Model 1300 instrument equipped with a 40x40x120 mm semi-octagonal cell. Measurements were made at 25°C with light of wavelength 463 nm using 25μM steroid solutions of varying water-co-solvent compositions which had been shaken thoroughly and then kept at 25° for 2 h. The excess turbidity values for each solution were calculated by the standard procedure (7).

The critical micelle concentration (CMC) of cholest-4-en-3-one (2e) in 75% aqueous methanol was determined by the above geñëral procedure on 8-70μM solutions.

## RESULTS

As stated in the Introduction, the initial studies on $\Delta^5$-3-ketosteroid aggregation had shown that complete solvation of 1a and 1e could be effected by adding methanol to the aqueous solutions. Accordingly, the propensities of the $\Delta^5$-3-ketones 1a-e to form micelles in aqueous solutions were first measured in terms of the minimum methanol concentrations required to effect their complete solvation.

The acid-catalyzed isomerizations of each of 1a-e were surveyed in aqueous solutions of increasing methanol content. 25μM steroid solutions were selected for all the kinetic assays on the basis of the convenient rates of change of optical density they provided. As expected, the data obtained in dilute aqueous methanol gave the curved, multiple-order $\log_{10}\Delta OD$ vs t plots indicative of aggregation. However, as the methanol contents of the solutions were raised, the lines became progressively

straighter until at the respective critical methanol concentrations, the straight line pseudo first-order plots diagnostic of complete substrate solvation were observed. The results obtained (summarized in Table I) showed that, as anticipated the more hydrophobic the C-17 substituent of compounds 1a-e the more stable the micelles become and the higher the minimum proportion of methanol required to effect their disaggregation. That the observance of a pseudo first-order acid-catalyzed isomerization plot was a reliable criterion for defining solvent conditions ensuring complete solvation of each steroid was confirmed by light scattering (8) experiments on the corresponding $\Delta^4$-3-ketones 2a-e. The effects of methanol on the turbidities of aqueous solutions of 2a-e are represented in

Table I. Correlation between the minimum methanol concentrations required to disaggregate micelles of 1a-e and 2a-e in aqueous solutions with the hydrophobicity of the C-17 substituent.

| C-17 Substituent | % Methanol for Complete Solvation | |
|---|---|---|
| | From Acid-Catalysis of 1 | From Light Scattering of 2 |
| H | 27[a] | 32 |
| $CH_3$ | 30 | 30 |
| $CH_2CH_3$ | 35 | 35 |
| $CH(CH_3)_2$ | 50 | 46 |
| $C_8H_{17}$ | 75[a] | 78 |

All measurements were performed on 25μM aqueous solutions at 25°. [a]cf. reference 2

Figure 1. The solvent compositions at which zero turbidities are first observed represent the minimum concentrations of methanol required to achieve complete solvation of the 25μM solutions of 2a-e. These values are recorded in Table I and it can be seen that for each $\Delta^4$- and $\Delta^5$-3-ketone having the same C-17 substituent, the minimum methanol concentrations determined by the light-scattering method are in close agreement with those derived by the acid-catalysis technique. Although each pair of compounds 1 and 2 are very

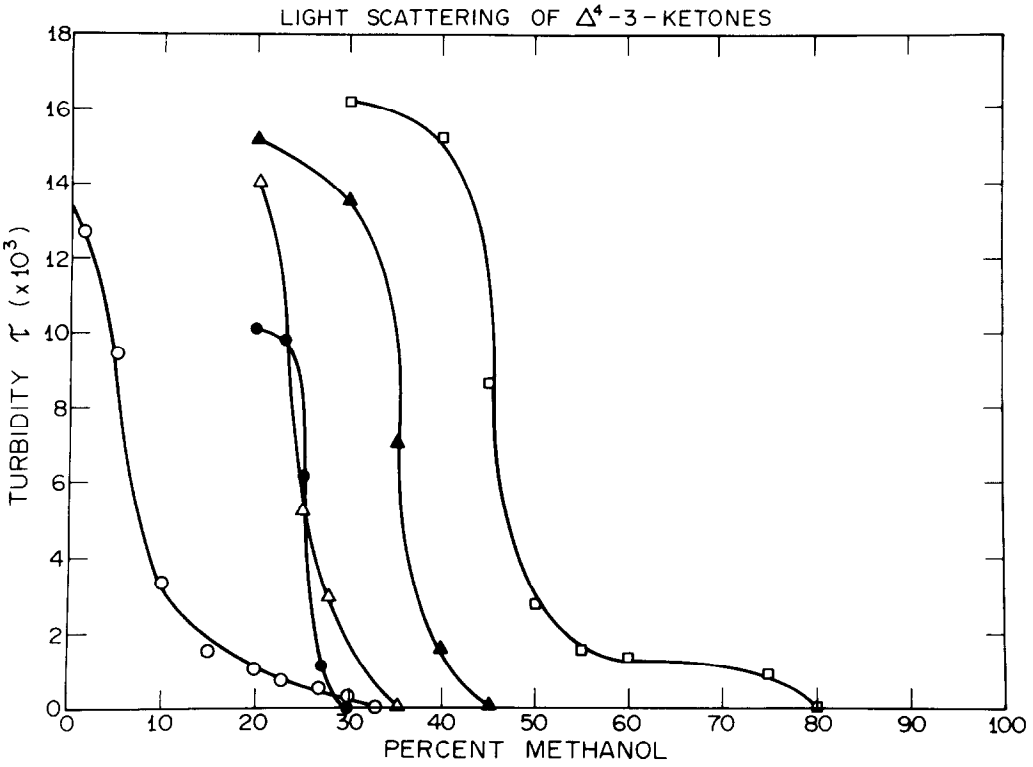


Figure 1. Effects of methanol on the light scattering properties of the $\Delta^4$-3-ketones 2a (○), 2b (●), 2c (△), 2d (▲) and 2e (□). The turbidity measurements were made on 25μM aqueous solutions at 25°.

similar in their aggregation properties, an exact correspondence of the critical methanol compositions would not be anticipated in view of the slight structural differences between the isomers.

The turbidity - solvent relationships depicted in Figure 1 and the data of Table I indicate that in the solvent composition required for zero turbidity or first-order kinetics, the CMC of each steroid is ∿25μM. However, since in micelle studies it is more conventional to determine CMC values by varying the substrate concentration in a solvent of fixed composition, a representative determination of the CMC of cholest-4-en-3-one (2e) in 75% aqueous methanol was carried out in order to confirm that our steroids were behaving in a typical micelle manner. The turbidity vs concentration plot obtained (Figure 2) was of the form often encountered in micelle studies (9) and the observed CMC of 26μM is in excellent agreement with the ∿25μM value predicted.

Since the methanol concentrations required to fully disrupt micelles of 1a-e were too high to permit any meaningful study of their $\Delta^5$-3-ketoisomerase specificity, a survey of other uv-transparent solvents was performed in the hope that complete solvation could be achieved at lower organic solvent concentrations. 20-Methylpregn-5-en-3-one (1d), with its C-17 group of median hydrocarbon character, was

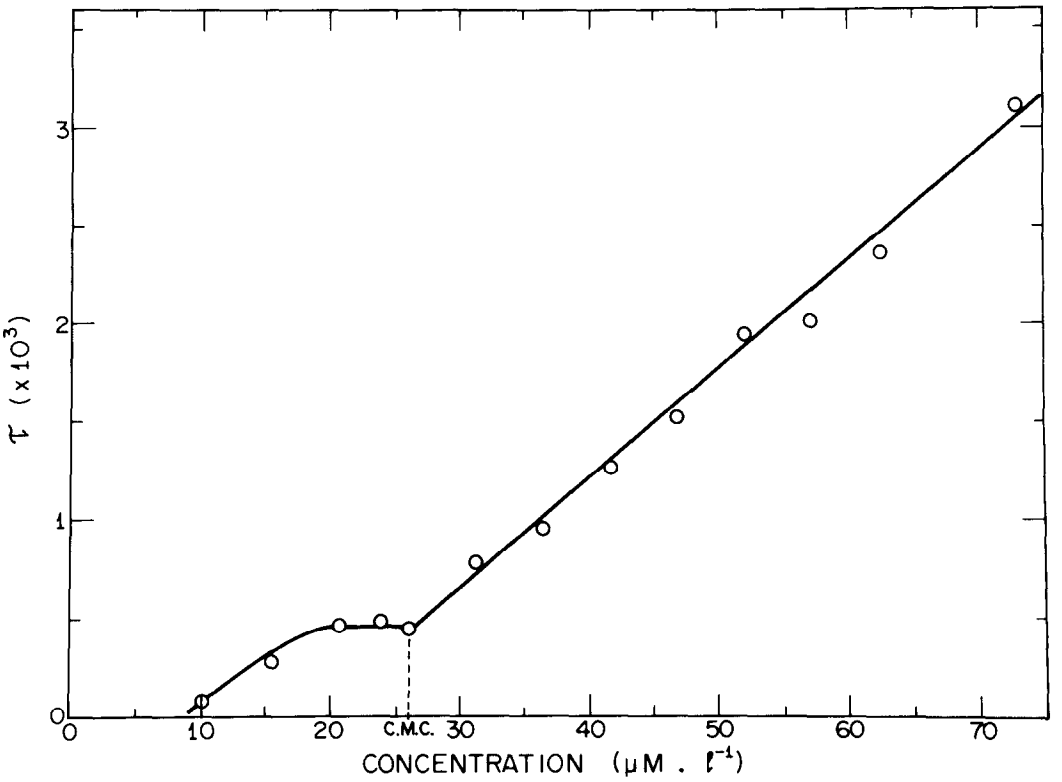


Figure 2. Determination of the critical micelle concentration of cholest-4-en-3-one (2e) in 75% aqueous methanol.

selected as the model substrate and the solvents evaluated and the minimum concentrations required for first-order acid-catalysis kinetic plots are depicted in Table II. The results showed that all the solvents investigated were more effective than methanol in causing micelle break-up with 1-propanol being by far the most satisfactory.

For the homologous straight-chain alcohols methanol, ethanol, and 1-propanol, a linear correlation was observed

Table II. Acid-catalyzed isomerization of 20-methylpregn-5-en-3-one (1d) in different solvents.

| Solvent | % Required for 1st-order plots[a] |
|---|---|
| Methanol | 50 |
| Diglyme | 45 |
| Dioxane | 40 |
| Glyme | 37 |
| Ethanol | 35 |
| 2-Propanol | 27 |
| Allyl Alcohol | 25 |
| Tetrahydrofuran | 23 |
| 1-Propanol | 20 |

[a]Measurements were performed on 25μM aqueous solutions at 25°.

between the number of methyl or methylene groups in the alcohol and their respective concentrations required to disaggregate micelles of 1d. As Figure 3 shows, this linear relationship was found to be general for each of the substrates 1a-1e. Each increase in the hydrocarbon character of the C-17 substituent caused a decrease in the slope of the line observed. Considerable information on the nature of the steroid micelles can be deduced from these data since in a homologous surfactant or solvent series, the extent of hydrophobic bonding is generally reflected in the free energy of micellization per methylene group, $\Delta G_{CH_2}$ (10-13). Accordingly, ethanol-methanol and 1-propanol $\Delta G_{CH_2}$ values were calculated (11,12) for each of the $\Delta^5$-3-ketones 1a-1e. The free energy energy differences, which are recorded in Table III, approximate those estimated

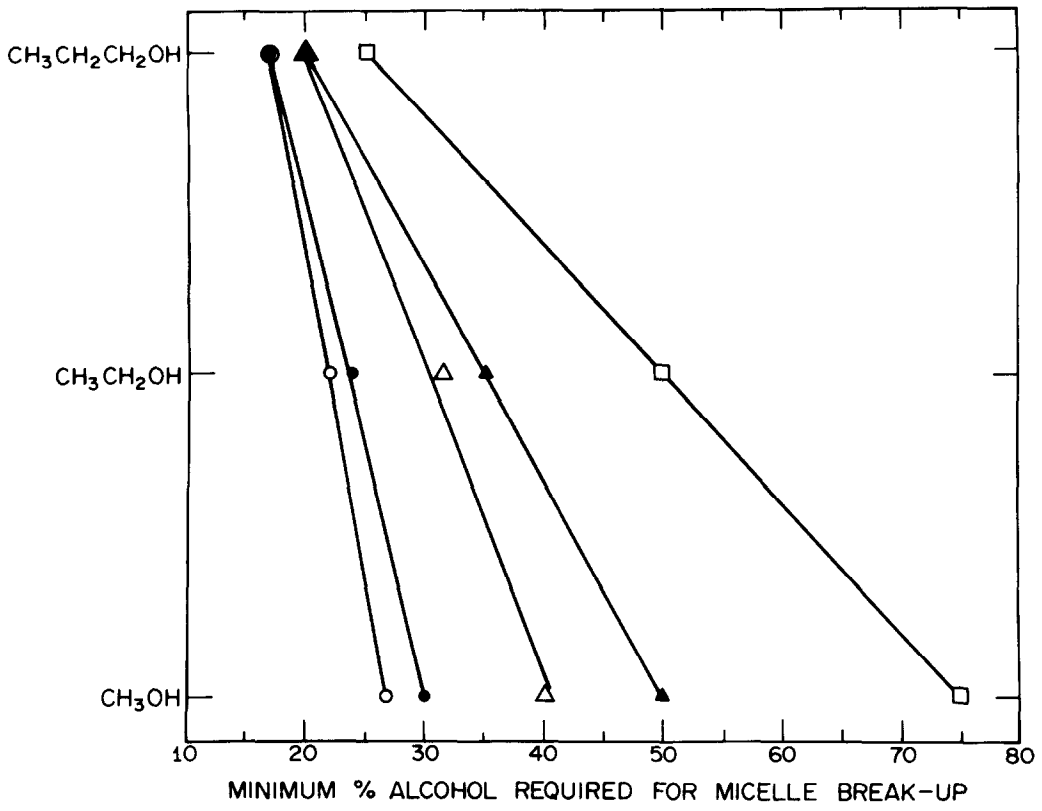


Figure 3. Linear correlation between the number of methyl or methylene groups of the homologous alcohols methanol, ethanol and 1-propanol with the proportion required to achieve disaggregation of micelles of 1a(○), 1b(●), 1c(△), 1d(▲) and 1e(□). The alcohol percentages required were determined by the acid-catalyzed isomerization technique on 25μM solutions at 25°.

for lecithin aggregates for the same series of alcohols (11).

In order to ascertain whether or not the C-17 groups were exerting a specific effect on the aggregation tendencies, similar $\Delta G_{CH_2}$ calculations (11,12) for the pairs of homologous substrates 1a and 1b, and 1b and 1c, were also carried out. However, the $\Delta G_{CH_2}$ values (Table IV) did not

Table III. $-\Delta G_{CH_2}$ Values for the interaction of the homologous alcohols methanol, ethanol, and 1-propanol with micelles of 1a-e.

| Compound (C-17 group) | $-\Delta G^a$(cal.mole$^{-1}$) EtOH-MeOH | $-\Delta G^a$(cal.mole$^{-1}$) 1-PrOH-EtOH |
|---|---|---|
| 1a (H) | 336 | 319 |
| 1b ($CH_3$) | 354 | 363 |
| 1c ($CH_2CH_3$) | 356 | 435 |
| 1d ($CH(CH_3)_2$) | 424 | 497 |
| 1e ($C_8H_{17}$) | 451 | 581 |

[a]Calculated (11,12) from the data summarized in Figure 3.

show any consistent pattern and their individual values were generally small.

Table IV. $-\Delta G_{CH_2}$ Values for the interactions of micelles of the homologous pairs of $\Delta^5$-3-ketones, 1a,b, and 1c,d in methanol, ethanol, and 1-propanol.

| Alcohol | $-\Delta G^a$(cal.mole$^{-1}$) $H-CH_3$ | $-\Delta G^a$(cal.mole$^{-1}$) $CH_3-CH_2CH_3$ |
|---|---|---|
| Methanol | 62 | 170 |
| Ethanol | 30 | 168 |
| 1-Propanol | 0 | 96 |

[a]Calculated (11,12) from the data summarized in Figure 3.

## DISCUSSION

Our initial studies (2) on $\Delta^5$-3-ketone substrates of the isomerase from P. testosteroni had indicated that the facility with which micelles formed would become progress-

ively greater as the hydrophobic character of the C-17 group was increased. The series of substrates 1a-e of gradually increasing hydrophobic character were therefore synthesized (3) in order than a more definitive study of the influence of apolar C-17 substituents might be carried out. Since the proportion of methanol required to disrupt the micelles had appeared (2) to provide a measure of the aggregation tendency, the effect of methanol on aqueous solutions of 1a-e was evaluated first.

The results obtained (Table I) reflected a progressively increasing stability of the substrate micelles as the hydrophobic character of the C-17 side-chain increased. However, since the isomerization kinetics technique represents an indirect approach to micelle detection, its validity was checked by the more traditional light-scattering method (8). The light-scattering determinations were carried out on the conjugated ketones 2a-e rather than on their βγ-unsaturated isomers since it was known that significant spontaneous isomerization of the latter compounds would occur during the relatively lengthy procedures involved. The data obtained (Figure 1 and Table I) confirmed that the minimum methanol concentrations required for first-order kinetic plots using the acid-catalysis technique did represent the conditions under which complete disaggregation of the aggregates was first achieved. The turbidity-%methanol relationships observed also provided a clearer picture of the effect of methanol on the steroidal aggregates. The curves in Figure 1 show that methanol is extremely

effective in causing micellar disaggregation since its addition in small amounts causes large decreases in turbidity for each of 2a-e. The correspondence of the %methanol data of Table I and the close agreement between the 26μM value for the CMC of 2e in 75% aqueous methanol obtained by a conventional light-scattering procedure (Figure 2) with the ∿25μM estimated for 1e by the acid-catalysis technique provided verification that the latter kinetic method was a reliable one for monitoring micelle formation and break-up (14).

While the effects of organic solvents on steroid micelles have received little attention, the properties of surfactant micelles in solutions of organic solvents are well documented (8,10,15). For most solvents, the situations are not simple and the overall effect is a composite of their influence on the water structure in the immediate vicinity of the micelle, their ability to form mixed micelles by penetration, and of the consequences of altering the dielectric constant of the medium (16). Accordingly, in view of the difficulties involved in predicting the effect of a given solvent on the stabilities of micelles of the $\Delta^5$-3-ketones 1, a survey of a series of solvents was carried out in the hope that more suitable disaggregation conditions could be found for the subsequent enzyme studies intended. From the survey conducted

on 1d, it is apparent (Table II) that micelle stabilization by solvent penetration (8,17,18) cannot be significant since 1-propanol, 2-propanol, and ethanol disaggregate more effectively than methanol. Furthermore, the dielectric constant of the medium is clearly not of overriding importance since dioxane (ε 2.2) and methanol (ε 32.6) have almost equivalent effects on the micelles of 1d.

The most reasonable rationale for the solvent survey results is that the ability of a solvent to disrupt the steroid micelle is a reflection of its power to solvate the hydrophobic monomeric species (19). On this basis solvents possessing the highest ratio of hydrophobic to hydrophilic areas should disrupt the aggregates most effectively viz. higher members of a homologous series more readily than lower, straight-chain compounds more readily than their branched isomers, and acyclic structures more readily than their cyclic isomers. A re-examination of Table II reveals that the data are consistent with this view. Ascension through the series methanol, ethanol, and 1-propanol decreases the proportion of alcohol necessary for complete solvation. Moreover, the branched-chain 2-propanol is less effective than its straight-chain isomer 1-propanol, while the latter is superior to the homologous cyclic compound tetrahydrofuran.

Of particular interest was the observation that for the homologous alcohols methanol, ethanol, and 1-propanol,

there was a linear correlation between the number of methylene equivalents and the respective concentrations required for complete disaggregation of la-e (Figure 3). Linear relationships of this type do not appear to be common although an approximately linear relationship of the above type has been observed for the disaggregation of lecithin micelles (11). A further indication of the nature of the hydrophobic interactions of the above series of homologous alcohols with la-e was provided by the differences in the free energies of micellization per methylene group of the solvent (10-13, 20). The $\Delta G_{EtOH-MeOH}$ and $\Delta G_{1-PrOH-EtOH}$ values (Table III) decrease progressively as the hydrocarbon character of the C-17 group increases and when the side chain is $C_8H_{17}$, the values approach the range observed for typical micelles (10). While these data might be mistaken as evidence for a specific hydrophobic interaction between the solvent and the C-17 alkyl groups, it should be emphasized that the decreases in $\Delta G_{CH_2}$ for each additional side chain extension are small. It must therefore be concluded that no specific influences are attributable to the C-17 groups and that the degree of solvation of each molecule is dependent on its overall hydrophobic character. This conclusion was confirmed by the observation that the $\Delta G_{CH_2}$ values calculated for the C-17 homologues la-c were small and apparently random (Table IV). Nevertheless, the fact that the magnitudes of the $\Delta G_{CH_3-C_2H_5}$ values are significantly lower than those of $\Delta G_{H-CH_3}$ for each

solvent must reflect an increased hydrophobic interaction due to the lengthening of the C-17 hydrocarbon chain. The total data obtained suggest that the solvation of the steroid micelles by alcohols and other organic solvents occurs by a process analogous to that postulated for the break-up of lipid aggregates by non-penetrating alcohols for which it has been suggested that adsorption of the alcohol at the hydrocarbon-water interface of the lipid leaflets is involved (12).

In contrast to the information available on relatively hydrophilic steroids such as the bile acids (21) surprisingly little is known regarding the structure of aggregates of hydrophobic steroids. The above investigations suggest that la-e, as predominantly hydrophobic molecules possessing a hydrophilic ketone "head" group, are behaving somewhat like non-ionic surfactants although the detailed structures of the respective micelles are undoubtedly different (8,22,23). On the basis of the current evidence, and examination of molecular models, we consider that a reasonable speculation regarding the structures of the steroid micelles is as depicted in Figure 4 with the molecules stacked to allow maximum overlap of the hydrophobic steroid skeletons. The number of molecules per micelle will naturally vary with the solvent composition but it seems probable that more than the two or three suggested for cholesterol associates (24,25) will be involved in aqueous solutions. However, as implied earlier, it is possible that the steroid aggregates may also

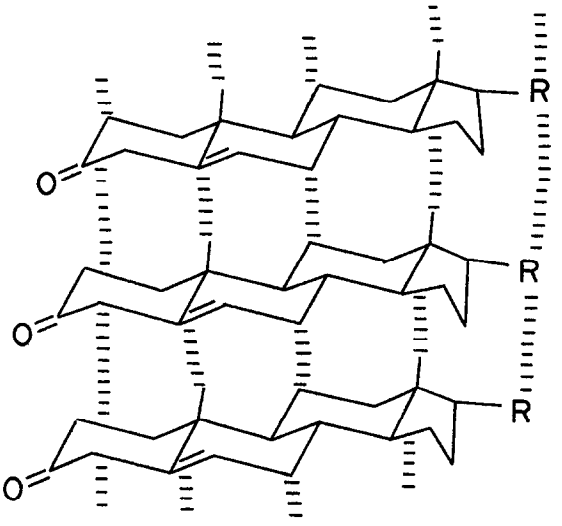


Figure 4. Proposed structural form of the micelles of la-e in aqueous solutions.

resemble the bimolecular leaflets of lecithin micelles (11,12).

Through this investigation, acid-catalyzed isomerization of $\Delta^5$-3-ketones has demonstrated itself to be a convenient procedure for monitoring their micellar aggregation tendencies and properties. Since $\beta\gamma$-unsaturated ketones are readily preparable (2,3) this kinetic procedure should be generally applicable to studies on micelles of variously substituted steroids and other compounds and will often be more convenient to use than the hitherto more commonly employed physico-chemical techniques (8,15). Furthermore, it has been found to be more sensitive than any of the latter (26) and additional illustrations of its application during specificity studies on la-e and other

C-17-substituted substrates of the $\Delta^5 \rightarrow \Delta^4$-3-ketoisomerase of P. testosteroni will be reported shortly.

ACKNOWLEDGMENT

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