creted, do not penetrate biological membranes (16). It is, however, known that specialized transport mechanisms for endogenous glucuronides exist in the kidney and the liver (17). In view of the present findings relative to erythrocyte sequestration, it is tempting to suggest that the same or similar transport mechanisms may be involved in actively transporting the conjugated metabolites across the erythrocyte and possibly even other biological membranes.

Supporting this contention is the ubiquitous existence of β-glucuronidase in tissues, the reason for which remains unexplained. Could it be that the active transport system controlling the transfer of glucuronides across the cell membranes is coupled with the β-glucuronidase system? It is conceivable that following the active transport of the conjugated molecules, for example at the biophase, there is an instantaneous hydrolysis and thus liberation of the drug or metabolite molecules which may then exert their pharmacological effect. Studies now in progress are aimed at investigating the validity of the belief that conjugation may have important biological significance in membrane transport of pharmacologically active molecular species. The preliminary data on human subjects indicate that the urinary elimination of the conjugated chlorpromazine metabolites was significantly inhibited by penicillin which is known to be actively secreted by the tubular cells.

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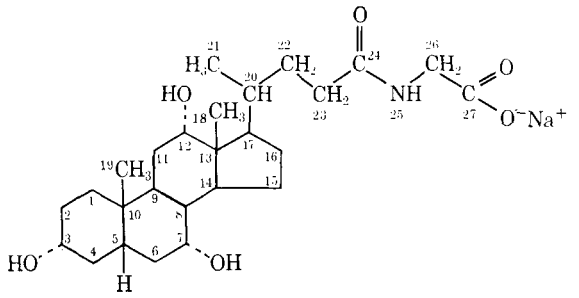
Micelle Formation and Testosterone Solubilization by Sodium Glycocholate

LEO MARTIS*, NATHAN A. HALL*, and ARVIND L. THAKKAR†▲

Abstract □ Surface tension and light-scattering studies on aqueous solutions of sodium glycocholate gave CMC values of 0.0129 and 0.0138 mole/l., respectively. Light-scattering studies in 0.1 *M* NaCl gave a weight-average micellar weight of about 1950, indicating that four monomers of glycocholate aggregate to form the primary micelle. Preferential broadening of the angular methyl signals in the NMR spectra suggests that glycocholate micelles are formed by back-to-back hydrophobic association of the steroid nuclei. The signal due to the glycine methylene protons in the hydrophilic region of the molecule showed little broadening. Solubility studies showed that testosterone is solubilized by glycocholate below the apparent binary CMC. The solubilizing capacity is quite low. The complex association between testosterone and glycocholate suggests mixed micelle formation.

Keyphrases □ Sodium glycocholate—micellar properties, determination of CMC, solubilization of testosterone □ Micelle formation, sodium glycocholate—determination of CMC, solubilization of testosterone □ Testosterone—solubilization by sodium glycocholate □ Solubilization—testosterone by sodium glycocholate

Pharmaceutical systems have involved surfactants for many years. Such agents show grossly similar chemical characteristics, typified by hydrophobic and hydrophilic portions of their molecules. Bile salts, however, differ both in chemical structure and colloidal behavior from the conventional surface-active molecules. The formation of micelles in the case of bile salts is not as abrupt as in the case of ordinary association colloids. Ekwall *et al.* (1) showed that the micelle formation for different bile salts passes through introductory association stages. The concentrations at which these steps take place are determined mainly by the number of hydroxyl groups in the bile acid molecules. Small (2) and Carey and Small (3) studied the properties of bile salt solutions and reported that trihydroxy bile salts form very small micelles at all concentrations while dihydroxy bile salts



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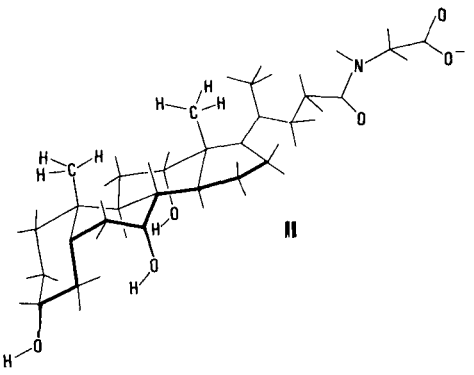

II

form very small micelles at low concentrations but much larger micelles at high concentrations.

Surface tension measurements have not been used widely to study bile salt solutions. Early workers failed to find well-defined CMC's, probably because bile salts free from impurities were difficult to obtain. Recently, however, Kratohvil and DelliColli (4) reported a careful study of solutions containing either sodium taurodeoxycholate or sodium glycodeoxycholate by surface tension measurements, supplemented by light-scattering and refractometric data. They found these dihydroxy bile salts to have quite low aggregation numbers, which increased about sixfold in the presence of added electrolyte. These researchers concluded that the observed micellar properties were very much like those of other typical anionic detergents.

This study was undertaken to investigate the micellar properties of the trihydroxy bile salt, sodium glycocholate (I), by surface tension and light-scattering methods and to supplement the quantitative data thus obtained with NMR measurements of a qualitative nature. NMR spectroscopy has been applied in recent years to the study of micelle formation. This technique can provide an insight at the molecular level regarding how certain sites in the monomers interact to give rise to micellar aggregates. Small *et al.* (5) used NMR spectroscopy with several unconjugated bile salts. They found that, in aqueous solutions, signals due to the two angular methyl groups undergo appreciable broadening as the bile salt concentration is increased, indicating thereby that bile salt micelles are formed by back-to-back association of the steroid nuclei. Spectral studies with sodium glycocholate offer an improvement in that there are probes in both the hydrophobic and the hydrophilic regions (II). The two angular methyl groups, particularly the one at position 18, and the methylene protons in the glycine portion of the molecule can be observed for selective linewidth broadening.

A second purpose of this study was to investigate the solubilization of a steroid by the steroidal surfactant, sodium glycocholate. Such investigation could be of biological and theoretical importance since bile salts have been shown to be unusual in their micellar properties. Some differences also might be expected in solubilized systems. Previous studies (6, 7) showed that the aqueous solubility of steroids can be enhanced by bile salts. In the present study, NMR spectra of glycocholate solutions saturated with testosterone were also examined for evidence of the location of solubilized testosterone.

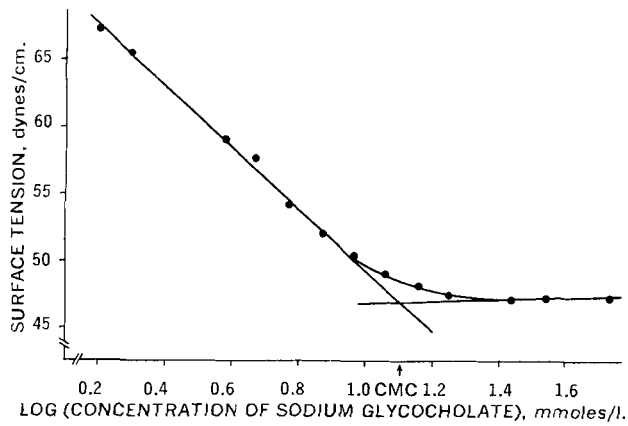

Figure 1—*Surface tension of sodium glycocholate solutions at 25°.*

EXPERIMENTAL

Materials—Sodium glycocholate[1], testosterone[2], and reagent grade sodium chloride and ethanol were used as received. Double-distilled water was used for surface tension, light-scattering, and solubility studies. Deuterium oxide[3] (99.7 mole%) was used for NMR studies.

Surface Tension Measurements—Surface tension at the air–water interface was measured with a surface tensiometer[4], employing a platinum blade (perimeter 5 cm.) by the Wilhelmy plate principle (8). Between the measurements, the blade was rinsed thoroughly and heated to incandescence in a flame. The measurements were made at a constant temperature by circulating water at 25 ± 0.1° around the solution container.

Light-Scattering Measurements—A light-scattering photometer[5] was utilized for measurement of turbidity at a wavelength of 436 nm. The working standard method of Brice *et al.* (9) was used. Square cells (30 × 30 mm.) were employed in determining the scattering intensities at an angle of 90°. The apparatus was not equipped with a thermostat, and all measurements were done at an ambient temperature of about 25°.

The refractive index increments, $\Delta n/C$, were determined with a differential refractomer[6] at 25 ± 0.1° at 436 nm. The apparatus was calibrated by the use of standard sucrose solutions (10).

NMR Studies—Solutions of various concentrations of sodium glycocholate were prepared in deuterium oxide, with and without 0.1 *M* NaCl. NMR spectra were recorded on a spectrometer[7]. External tetramethylsilane was used as the source of a lock signal. The complete spectra were initially scanned, and then signals of in-

[1] A grade, Calbiochem, Los Angeles, Calif.
[2] Nutritional Biochemical Corp., Cleveland, Ohio.
[3] Merck Sharp and Dohme, Montreal, Canada.
[4] Rosano, Federal Pacific Electric Co., Newark, N. J.
[5] Brice-Phoenix No. 560, Phoenix Precision Instrument Co., Philadelphia, Pa.
[6] Brice-Phoenix No. 430, Phoenix Precision Instrument Co., Philadelphia, Pa.
[7] HA-100, Varian Associates, Palo Alto, Calif.

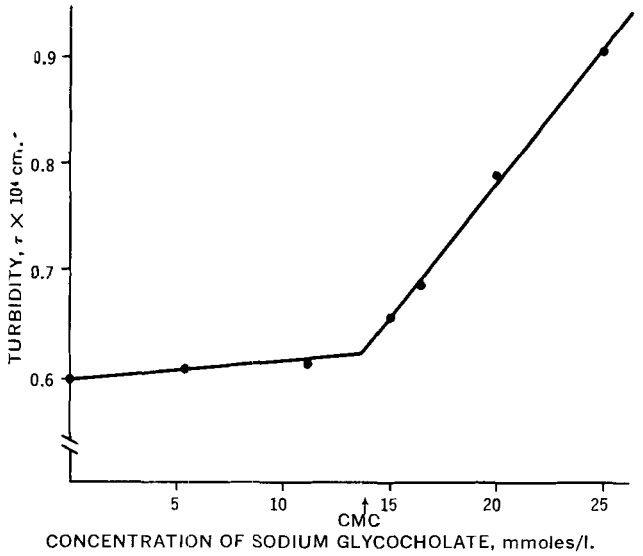

Figure 2—*Turbidity of sodium glycocholate solutions at 25°.*

terest were recorded three times to obtain the linewidth at half-height. The linewidths thus measured were estimated to be accurate to ±0.1 Hz. The normal operating probe temperature was 30 ± 1°.

Testosterone Solubility Determinations—To a series of screw-cap vials, each containing 10 ml. of sodium glycocholate solution of known concentration, sufficient testosterone was added to ensure an excess at equilibrium. The vials were shaken in a water bath shaker at 25 ± 0.1° for 48 hr. As observed previously (11), solubility equilibrium was reached after an initial supersaturation because of the conversion of the more soluble, metastable anhydrous form of testosterone to the hydrate form (12). In glycocholate solutions this conversion was essentially complete in 48 hr., since there was no detectable change in the equilibrium solubility value after this time. Upon equilibration, the contents of the vials were filtered through 0.45-μ Millipore disks and were analyzed spectrophotometrically by relating the peak absorption of the sample to that of a standard testosterone solution in ethanol. Peak heights were taken from spectral recordings from 300 to 200 nm., since in ethanol the peak wavelength shifted about 8 nm. bathochromically. Samples were diluted with ethanol as necessary. Measurement of areas under the peak was not practical due to interference by the shoulder of the glycocholate peak. A spectrophotometer[8] and matched cylindrical silica cells of 10-mm. light pathlength were used.

RESULTS AND DISCUSSION

CMC—Figure 1 shows that the surface tension decreases rapidly at low concentrations of sodium glycocholate and remains fairly constant above the CMC. Accordingly, the CMC is determined from the inflection point of the surface tension *versus* log concentration curve. A CMC value of 0.0129 mole/l. is obtained from Fig. 1 for sodium glycocholate. This value is within the general range of CMC values suggested (13) for trihydroxy bile salts. Benzonana (14) determined the CMC of sodium deoxycholate by surface tension measurements and observed a minimum surface tension at the CMC. This minimum, which cannot be explained thermodynamically, is probably due to the impurities that dissolve in the micelle (15). The absence of such a minimum surface tension at the CMC in the present study indicates the lack of surface-active impurities in the sample used (13).

The determination of CMC by light scattering depends on the fact that the scattering power of simple ions and monodisperse molecules is negligible compared to that of the same ions in the micellar form (16). Thus, the CMC can be determined from a plot of turbidity against concentration. Figure 2 represents such a plot for aqueous solutions of sodium glycocholate, from which a CMC value of 0.0138 mole/l. is obtained. This CMC is in the same range

[8] Cary model 14.

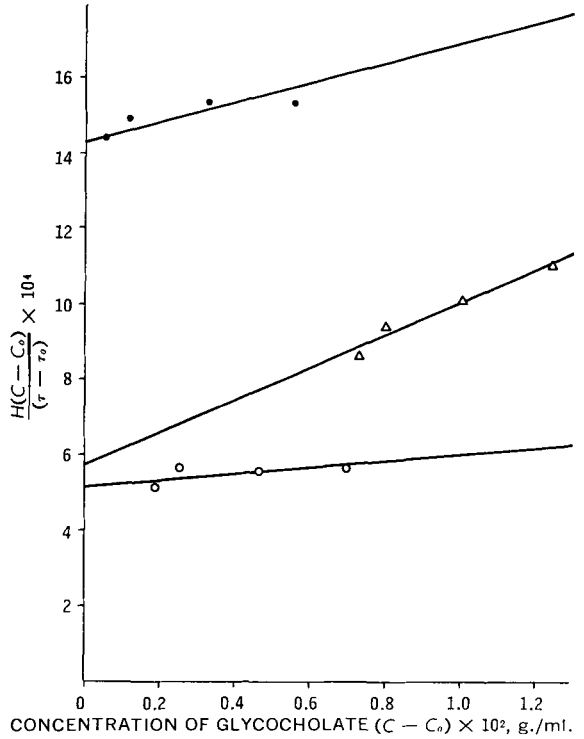

Figure 3—*Debye plot for sodium glycocholate solutions. Key: ●, sodium glycocholate; ○, sodium glycocholate in 0.1 M NaCl; and Δ, sodium glycocholate and testosterone in 0.1 M NaCl.*

as the CMC obtained from surface tension measurements. The agreement of CMC values is an indication of the efficiency of the procedure used in clarifying solutions for light-scattering experiments.

Micellar Weight Determinations—To estimate the weight-average micellar weight, the light-scattering results are plotted according to the usual practice originated by Debye (16). Figure 3 represents such a plot of $H(C - C_o)/(\tau - \tau_o)$ *versus* $(C - C_o)$, where H is a constant, C_o is the CMC in grams per milliliter, and τ and τ_o are the turbidities at concentrations C and C_o, respectively, in cm.$^{-1}$. The constant H is given by:

$$H = \frac{32\pi^3 n_o^2[(n - n_o)^2/C]}{3\lambda^4 N} \qquad \text{(Eq. 1)}$$

where n_o is the refractive index of the solvent, n is the refractive index of the solution, λ is the wavelength of light used, and N is Avogadro's number. The intercept of the line $H(C - C_o)/(\tau - \tau_o)$ *versus* $(C - C_o)$ with the ordinate gives the reciprocal of the weight-average micellar weight. Calculation of dissymmetry values for all solutions measured indicated, as reported by Hermann (17), that no correction of the molecular weight for dissymmetry was needed.

If the micelles are uncharged or carry a small net effective charge, little difficulty in the interpretation of Debye's equation is encountered. If, on the other hand, the micelles carry a net charge, the Debye plot will have a slope, and the micellar weight calculated from such a plot will be less than the actual micellar weight. Figure 3 shows that the Debye plot for aqueous solutions of sodium glycocholate, which gives a micellar weight of 699.3, does exhibit an appreciable slope. The micellar weight thus obtained should, therefore, be corrected. According to Mysels (18), the reduction in micellar weight of charged micelles is about 10–20%. The results thus indicate that the micelles of sodium glycocholate are small, probably no more than two molecules in size.

It has been suggested (18) that to get correct micellar weights of aggregates in solution, the light-scattering studies should be done in the presence of added electrolyte. Figure 3 shows that the slope of the plot of values obtained in the presence of 0.1 *M* NaCl is practically zero, which suggests that the effective charge carried by micellar aggregates is negligible in the presence of sodium chloride. This decrease in charge is due to neutralization of the charge carried by sodium glycocholate micelles by physically bound

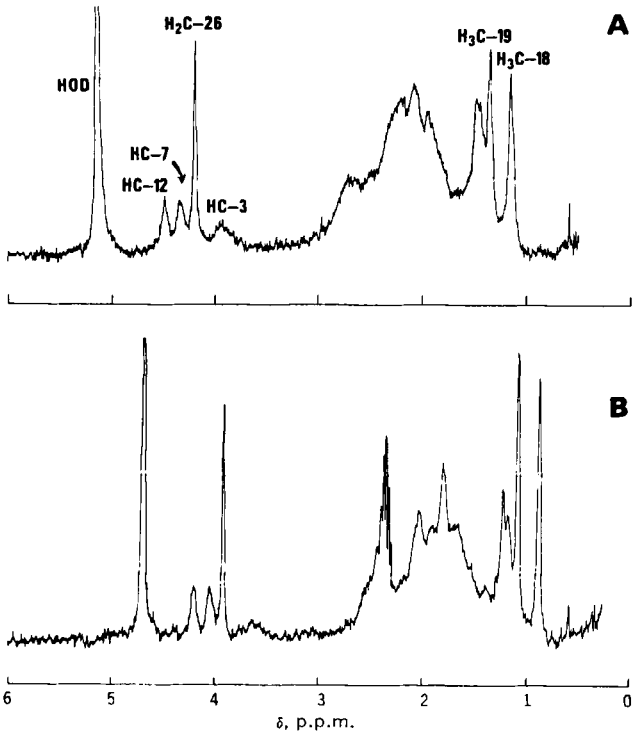

Figure 4—*NMR spectra of 0.1* M *sodium glycocholate in: (A) deuterium oxide and (B) 50% acetone-*d_6 *in deuterium oxide.*

counterions, Na^+, adsorbed on the micellar surface. The Debye calculations for micellar weight determination are, therefore, more reliable from data found in the presence of added electrolyte. The micellar weight thus determined is 1951, which corresponds to four molecules of sodium glycocholate per micelle. The increase in micellar weight in the presence of added electrolyte is in accord with polyelectrolyte theory (19). This observation is also consistent with other reports (2) regarding the effect of added electrolyte on the micellar weight.

NMR Studies—The high-resolution 100-MHz. NMR spectra of 0.1 *M* sodium glycocholate in deuterium oxide and in 50% acetone-d_6 (in deuterium oxide) are shown in Fig. 4. Signal assignments are based upon comparison with the spectra of cholic acid and other bile acids (5) and of other steroids (20). Because of their proximity to the electronegative oxygen and nitrogen atoms on the adjacent carbon atoms, the glycine methylene protons at C-26 resonate at about 4.2 p.p.m., considerably downfield from the methylene protons of the steroid nucleus.

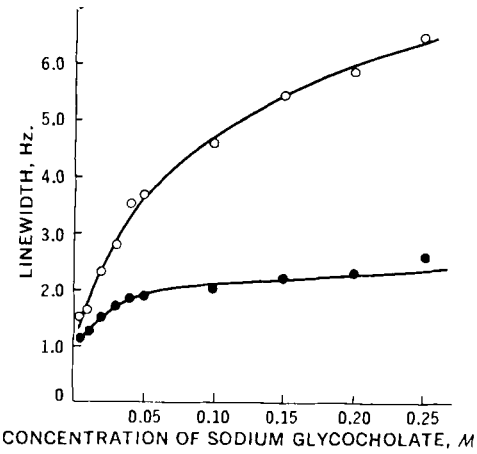

Figure 5—*NMR signal linewidths at half peak height* versus *concentration of sodium glycocholate. Key:* O, *C-18 methyl proton singlet; and* ●, *C-26 methylene proton singlet.*

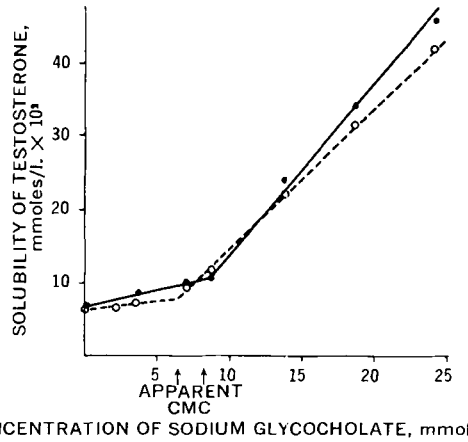

Figure 6—*Solubility of testosterone* versus *concentration of sodium glycocholate. Key:* ●, *without added electrolyte; and* O, *in presence of 0.1* M *NaCl.*

Signals of interest here are the sharp singlets of the methyl protons at C-18 and C-19 from the hydrophobic portion of the molecule and the singlet of the glycine methylene protons from the hydrophilic region of the molecule. At a concentration of 0.1 *M*, glycocholate in deuterium oxide should exist predominantly in the micellar form; whereas in 50% acetone-d_6, it should exist mostly in the monomeric form. This situation is reflected in the spectra. Signals due to the C-18 and C-19 methyl protons are relatively broad when the solvent is deuterium oxide, but these are quite sharp when the solvent is 50% acetone-d_6. The singlet, due to the glycine methylene protons, is quite sharp in both solvents.

The linewidths (at half of the signal height) of the C-18 methyl proton singlet and of the C-26 methylene proton singlets were determined as a function of the concentration of sodium glycocholate in deuterium oxide (Fig. 5). The linewidth of the signal from the hydrophobic region of the molecule undergoes greater broadening than does the signal from the hydrophilic portion of the molecule. Small *et al.* (5) made similar observations with several unconjugated bile salts. The linewidth increase is interpreted as being due to a specific effect and a general effect. The specific effect represents the restricted rotational freedom of the protons located in the hydrophobic part of the molecule, whereas the general effect reflects the increasingly ordered solute structure and a concomitant increase in the viscosity of the glycocholate solutions. The slight broadening of the C-26 protons can be attributed to this latter general effect. The greater preferential broadening of the C-18 methyl proton signal supports the proposal that micelle formation takes place by back-to-back association of the steroid nuclei.

In the presence of 0.1 *M* NaCl, the extent of signal broadening was greater and appeared to commence at a somewhat lower concentration. This behavior was expected in view of the established facts that the electrolyte would lower the CMC and that the micelles formed would be of larger aggregation number (2). The NMR method is too insensitive to allow determination of the CMC in this instance because concentrations higher than the CMC are required to record acceptable proton resonance spectra. Use of Fourier transform ^{13}C NMR might permit CMC determination.

Testosterone Solubilization—Figure 6 shows the relationship between the solubility of testosterone and the concentration of sodium glycocholate. The effect of 0.1 *M* NaCl on this relationship is also shown. Below the apparent CMC, there is a small but persistent increase in the solubility of testosterone. At glycocholate concentrations above the CMC, testosterone is solubilized at a higher rate. In the presence of 0.1 *M* NaCl, similar solubility behavior is seen but less testosterone appears to be solubilized both below and above the apparent CMC.

Solubilization of testosterone below the binary CMC of the glycocholate–water system suggests that testosterone somehow participates in the formation of mixed micelles or, more appropriately perhaps, in the formation of premicellar aggregates. Such behavior was noticed previously for testosterone with several surfactants (21, 22) and was attributed to the surface activity that

Table I—Solubilizing Capacity of Sodium Glycocholate for Testosterone at 25°

	Solubilizing Capacity—Moles Steroid/Mole Bile Salt		Reciprocal of Solubilizing Capacity—Moles Bile Salt/Mole Steroid	
	Without Electrolyte	With 0.1 *M* NaCl	Without Electrolyte	With 0.1 *M* NaCl
Below apparent CMC	0.0035	0.0023	285.7	357.1
Above apparent CMC	0.0220	0.0191	45.45	52.4

testosterone itself possesses. A closely related steroid, 19-nortestosterone, was also found to be solubilized below the apparent binary CMC's of sodium cholate and deoxycholate (7). Ekwall *et al.* (1) observed similar stepwise solubilization of cholesterol and bile acids as well as of some nonsteroidal solubilizates by bile salts. In all these cases, as in the present study, the amount of steroid solubilized increased with an increase in the concentration of the bile salt.

Testosterone solubilization at an increased rate above the apparent CMC of glycocholate, in agreement with the general features of micellar solubilization, suggests that as the number of glycocholate micelles increases, there is a proportional increase in the amount of testosterone solubilized. The solubilizing capacity of glycocholate for testosterone, calculated from the linear region of Fig. 6 above the CMC, is listed in Table I. When this solubilizing capacity value is compared with the previously obtained (7) solubilizing capacity values of cholate and deoxycholate for methyltestosterone, it appears that glycocholate ranks between these other two bile salts. For example, 1 mole of methyltestosterone is solubilized by 62 moles of cholate or by 32 moles of deoxycholate, whereas about 45 moles of glycocholate would be required to solubilize 1 mole of testosterone. It would seem from such low solubilizing capacities that the mechanism of solubilization previously proposed (7) for 19-nortestosterone, *i.e.*, solubilization within the interior of the micelle, is very likely not applicable here.

When NMR spectra of glycocholate solutions, without and saturated with testosterone, were examined, no additional broadening of 18 and 19 methyl signals was seen when the solutions contained solubilized testosterone. This observation would also suggest that testosterone is not solubilized within the nonpolar core formed by the hydrophobic backs of glycocholate molecules. When the UV spectral characteristics of testosterone below and above the CMC are examined, the wavelength of maximum absorbance shifts over a range of about 4 nm. toward lower wavelengths. Thus, the chromophore appears to be in a less polar environment upon solubilization. The absence of an appreciable increase in micellar weight when testosterone is solubilized (Fig. 3) in the presence of added electrolyte suggests that the testosterone is not incorporated within the glycocholate micelle. It seems likely that solubilization occurs through a complex association involving mixed micelles.

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