Behavior of Cholesterol Chlorobetainate in Aqueous Solution

By C. BOTRÉ, M. BRUFANI, and F. M. RICCIERI

Some physicochemical properties of cholesterol chlorobetainate in aqueous solution have been investigated. A critical concentration was detected by specific conductivity, refractive index, and activity coefficient determinations. Data are also reported on solubilization experiments with different polycyclic aromatic hydrocarbons. The differences between this compound and the most common ionic surfactants are discussed.

SEVERAL YEARS ago, Giacomello *et al.* prepared some steroid alcohol derivatives (esters with betaine hydrochloride) with very interesting physicochemical properties (1). Such compounds, in fact, pure or mixed with other esters of the same kind, gave gels with a water content 39 times the weight of solid substance.

X-ray diffraction as well as microscopic studies showed that the swelling of the crystal form takes place only in two dimensions, the third one being constant.

This study reports some physicochemical properties of one of these compounds (cholesterol chlorobetainate) which may contribute to the knowledge of the behavior of such compounds in aqueous solution.

Data are also reported on the solubilization of aromatic hydrocarbons in aqueous solutions of cholesterol chlorobetainate.

EXPERIMENTAL

Materials

The cholesterol chlorobetainate (CCB) was prepared according to Giacomello *et al.* (1), by reacting anhydrous trimethylamine with chloroacetylcholesterol at a temperature of $-10°$. All other reagents were analytical grade.

Procedure

Potentiometric Measurements.—Potentiometric analyses to determine the activity of chloride ions in solution were performed by using a radiometer potentiometer, model pHM4.

The Ag/AgCl electrode was directly dipped into the solution, while the calomel electrode was connected with the solution across a mixed liquid agar bridge, as described elsewhere (3). This was necessary to avoid the occurrence of flocculation at the tip of the electrode when simple agar bridges were employed. Continuous stirring of the solution was maintained by means of a glass rod, flattened at the tip, and running at constant speed into the solution.

Conductivity Measurements.—Conductivity measurements were carried out in a thermostatic bath at $25 \pm 0.01°$ using a WTW bridge model LF3.

Refractive Index Measurement.—Refractive index was measured by using a Rayleigh interference refractometer (Brice-Phoenix), equipped with monochromatic light sources of 546 and 436 mμ, respectively. Measurements were made at $20 \pm 0.5°$ according to Beckett *et al.* (2).

Solubilization of Polycyclic Aromatic Hydrocarbons.—The solubilization of naphthalene, anthracene, 3,4-benzpyrene, and naphthacene in aqueous medium at different concentrations of CCB was determined at room temperature.

All the solutions were equilibrated with very fine crystals of the hydrocarbons for 1 day.

After careful filtration through sintered-glass filters (Gooch G 5), volumes were extracted with known volumes of cyclohexane, and the hydrocarbon content in the organic phase was analyzed spectrophotometrically.

X-ray Diffraction Studies.—The mixed crystals were prepared by dissolving the CCB in water solution at a concentration of 4.5×10^{-3} *M* and then equilibrating in this solution very fine solid crystals of 3,4-benzpyrene. This suspension was kept at constant temperature for 1 day or more. After filtering, the clear solution was evaporated by standing at room temperature for several days. Small fluorescent crystals were thus obtained.

The finely ground crystals were put into a capillary and submitted to a Cu/Kα radiation.

Diffraction spectra of such crystals were recorded according to the Debye-Scherrer method.

RESULTS AND DISCUSSIONS

In Fig. 1 is shown the activity coefficient (γ-) of chloride ions of chlorobetainate in solution as a function of concentration of CCB. Figures 2 and 3 show, respectively, the specific conductivity (in ohm^{-1} cm.$^{-1}$) and the refractive index, in arbitrary units against molarity of CCB. From the trend of the plot γ- *versus* concentration as well as from the changes in specific conductivity and in refractive index, a break in the curves at the same concentration of 4.8×10^{-3} *M* is evident. By analogy with previous work (2, 3), the formation of aggregates might be inferred, like micelles in detergent solution.

To study the behavior of these aggregates in aqueous solution, solubilization experiments with sparingly soluble polycyclic aromatic hydrocarbons were performed. The results obtained were then

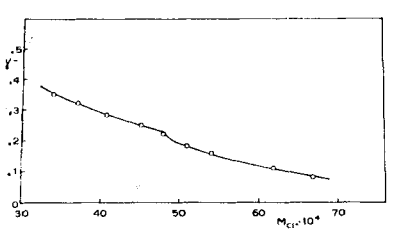

Fig. 1.—Activity coefficient γ- of chloride ions *vs.* the molar concentration of chlorobetainate in solution.

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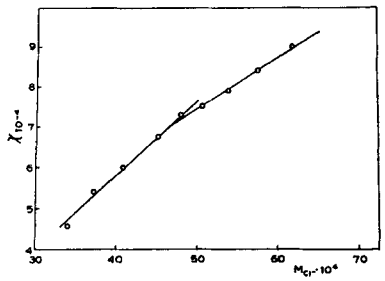

Fig. 2.—Specific conductivity, χ (in ohms^{-1} cm.$^{-1}$), *vs.* the molar concentration of chlorobetainate in solution.

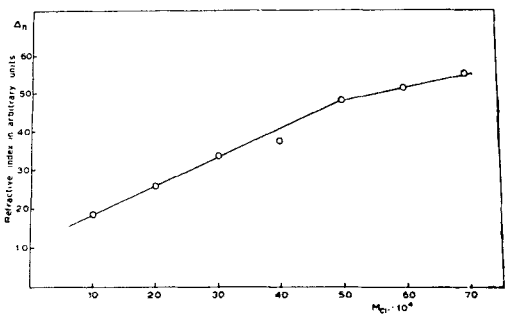

Fig. 3.—Changes of refractive index, in arbitrary units, *vs.* the molar concentration of chlorobetainate in solution.

compared with others of the same kind previously reported in the literature (4, 5).

It is known, in fact, that many attempts were made to prepare aqueous dispersions of association colloids containing polycyclic hydrocarbons with the aim of making their use in physiological experiments less difficult (6, 7). Generally in detergent solutions the solubilization of these hydrocarbons begins at the critical micelle concentration (CMC). The hydrocarbon is solubilized into the micelle in the polar part, and the absorption spectrum of the solubilized hydrocarbon does not differ significantly from the spectrum recorded in other organic solvents.

In Fig. 4 the absorption spectra of benzpyrene (BP) in cyclohexane (plot *1*), sodium lauryl sulfate (NaLS), 2×10^{-2} *M* (above CMC) (plot *2*), and CCB, at a concentration of 4.5×10^{-3} *M*, are compared. In Fig. 5 the diffraction photograph of BP (*A*), CCB (*B*), and the complex resulting from the interaction between CCB solution, 4.5×10^{-3} *M*, and BP (*C*) are compared. The complex CCB–BP is responsible for the differences observed both in the ultraviolet absorption and in the X-rays.

In this connection it should be noted that in the concentration range between 2 and 7×10^{-3} *M* CCB, the lowest concentration gives a diffraction pattern which is quite different with respect to both BP and CCB. By decreasing the ratio BP/CCB, *i.e.*, by increasing the CCB concentration, the CCB spectrum appears and is superimposed to the former.

On the other hand, if the experiment is performed with other aromatic hydrocarbons, *i.e.*, naphthacene, anthracene, and naphthalene, no shifts toward higher wavelengths are detected. Furthermore, the amount of naphthalene and anthracene dissolved in CCB is not significantly increased by higher CCB concentrations. Experiments in this direction are now in progress and will be published shortly.

Furthermore, from the ultraviolet spectra of BP in the above-mentioned media, it is evident that, both in cyclohexane and in NaLS, the spectra of BP do not show significant shifts of the maxima toward the higher wavelengths observed when CCB is used as a solubilizing agent. By analogy with previously reported solubilization experiments be-

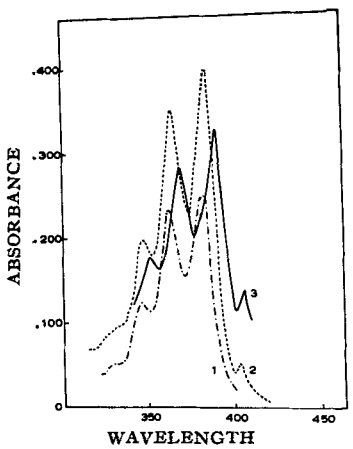

Fig. 4.—Absorbance of 3,4-benzpyrene in *1*, cyclohexane; *2*, sodium laurylsulfate in aqueous solution, 2.0×10^{-2} *M;* and *3*, cholesterol chlorobetainate in aqueous solution (4.5×10^{-3} *M*) *vs.* the wavelength (in mμ).

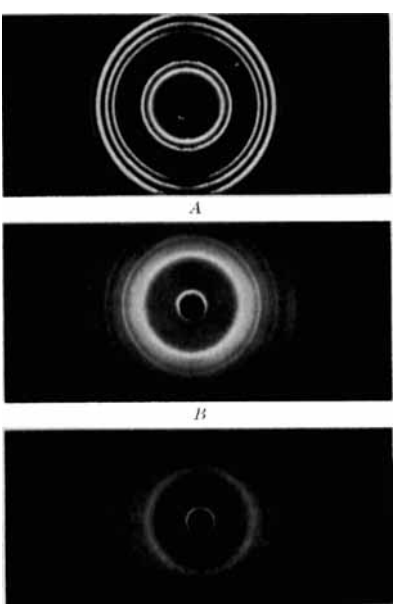

Fig. 5.—Diffraction photograph of: *A*, benzpyrene; *B*, cholesterol chlorobetainate; and *C*, complex.

tween BP and different purines or pyrimidines (8, 9), and where a similar shift takes place, it might be inferred that also in this case the interaction of the two molecular species, *i.e.*, the hydrocarbon and the purine or pyrimidine, leads to a formation of a molecular complex.

The difference between the solubilization of BP in CCB or in simple detergent solution above the critical concentration would perhaps be explained with an analogous mechanism. In the first case, however, more directional and specific forces would act between the solubilized molecule and the solubilizing agent.

Furthermore, when CCB is the host molecule, the amount of BP dissolved shows a different trend corresponding to the transition previously detected with conductivity, potentiometry, and refractrometry measurements.

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Identification of α- and β-Amanitin by Thin-Layer Chromatography

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A thin-layer chromatographic method using Silica Gel G plates developed in methanol–methyl ethyl ketone (1 : 1) proved suitable for the rapid detection of α- and β-amanitin in mushroom extracts. Application of the procedure to *Lepiota cepaestipes* and *Lepiota clypeolaria* indicated the amanitins were not present in these species.

EXISTING paper chromatographic methods for the detection of amanita toxins are generally unsatisfactory. Two very similar procedures are described in the literature. In the original method of Wieland and Schmidt (1), a methanolic extract of the fungus was spotted on S&S 2043b filter paper and the chromatogram developed for about 2 hr. with the upper phase of a solvent mixture comprising methyl ethyl ketone–acetone–water (20:2:5). If additional separation was desired, the chromatogram was developed in a second direction with ethyl formate–acetone–water (20:29:8). Visualization of α- and β-amanitin and phalloidin was effected with a number of reagents, especially diazotized sulfanilic acid and cinnamaldehyde–hydrochloric acid.

A slight modification of this procedure was developed by Block *et al.* (2). A methanol extract of the fungus which had been repeatedly evaporated to dryness to coagulate polypeptides was applied to 1 × 14 in. filter paper strips (S&S 2043b or Whatman No. 1) and formed for 2 hr. with a solvent mixture made up of methyl ethyl ketone–acetone–water–*n*-butanol (20:6:5:1). Spraying with 1% cinnamaldehyde in methanol and exposure to hydrochloric acid vapor caused the amanita toxins to appear as violet- or blue-colored spots.

As has been previously pointed out (3), these methods have definite drawbacks, and their application to fungal extracts requires considerable experience and definite precautions to avoid erroneous interpretation of the results. The spotting of large amounts of concentrated methanol extracts, as recommended by Block *et al.* (2), often gives streaked and distorted chromatograms, probably due to the presence of accompanying lipids. The Wieland and Schmidt (1) solvent mixture forms an emulsion which must be centrifuged prior to use, and neither it nor the modified system of Block *et al.* gives reproducible results. R_f values of the amanitins vary appreciably with the quantity spotted, and freshly prepared solvent mixture gives different values than solvent aged several days prior to use.

It therefore seemed advantageous to develop a procedure which would permit rapid identification of α- and β-amanitin with greater certainty. Thin-layer chromatography appeared to present a suitable method, and its application to the problem was subsequently undertaken.

EXPERIMENTAL

Chromatographic Procedure.—Several solvents, such as ethanol, methanol, and methyl ethyl ketone, and a number of solvent mixtures including methyl ethyl ketone–acetone–water–*n*-butanol (20:6:5:1), *n*-butanol–ethanol—water (4:1:1), methanol–diethylamine (20:1), methanol–methyl ethyl ketone–diethylamine (various proportions), and methanol–methyl ethyl ketone (1:1) were tested for their ability to separate α- and β-amanitin on thin-layer plates. Best results were obtained with plates prepared with Silica Gel G and developed with a solvent system composed of methanol–methyl ethyl ketone (1:1).

The plates were prepared by vigorously shaking

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