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Cholesterol Solubility in Lecithin–Bile Salt Systems

Abstract. *The method of sample preparation can markedly influence the rate of dissolution and attainment of supersaturated states of cholesterol. The equilibrium solubility of cholesterol, studied as a function of its physical state in a model bile system, is almost half that of previously accepted values. Slow attainment of the equilibrium state may have acted to bias previous studies. Extrapolation of our data to the clinical situation reveals that many persons considered normal by present standards actually possess bile that is supersaturated with respect to cholesterol and are thus potential gallstone formers.*

The solubility of cholesterol in bile salts (*1*), in phospholipids (*2*), in mixtures of both (*1, 3, 4*), and in human or animal biles (*4, 5*) has been the subject of many investigations. The data presented in these studies show a variation in the equilibrium solubility of cholesterol. Since choiesterol has been reported to exist in different crystalline forms, which vary in crystal lattice parameters and extent of solvation (*6*), it is conceivable that the differences in equilibrium solubility can be attributed to differences in the solid state properties of cholesterol.

We undertook studies to monitor the solubility of cholesterol as a function of its physical state in a lecithin–bile salt system. The bile salts (conjugated bovine) and lecithin (egg) were purified (*7*) and used in a constant molar ratio (3.5/1), designed to mimic their biliary ratio. Several different forms of [4-^{14}C]cholesterol were produced by crystallization from aqueous and nonaqueous solvent systems, vacuum drying, or simultaneous precipitation (coprecipitation) with lecithin and bile salt. X-ray diffractometry, infrared spectroscopy, microscopy, and thermal gravimetric and differential thermal analytic methods were used to characterize these materials. An excess (20 mg ml^{-1}) of the crystalline cholesterols was added to a sterile lecithin–bile salt micellar system (37°C, *p*H 7.4, 0.05*M* phosphate buffer). The cholesterol solubility in the coprecipitate system was followed after addition of the appropriate concentration of buffer. Solubility was monitored by liquid scintillation counting of a filtrate obtained by passage through a membrane system (Millipore HA and AP20).

The results in Figure 1 show that changing the physical state of cholesterol can markedly influence its dissolution characteristics. The coprecipitated cholesterol, existing in an amorphous state dispersed with the lecithin and bile salt molecules, dissolves readily due to the instantaneous formation of lecithin–bile salt–cholesterol micelles after addition of aqueous solvent. The rapidity of this process may be ascribed to the ease with which the amorphous state may be disrupted by the solvent, as well as to the fact that the rate of transport of the cholesterol to and into the micelle is effectively infinite because the micelle is formed around it. The anhydrous cholesterol dissolves at a much greater rate and obtains a higher metastable solubility level than does the hydrate. These higher levels can be reduced by the nucleation of the truly stable, lower-energy hydrous species, with subsequent relief of the supersaturation by precipitation. The rate of conversion is influenced by the presence of crystal growth poisons and the extent of supersaturation. Nucleation and growth of the hydrate from the anhydrous species become apparent after 5 days, while equilibrium solubility in this system is approached in approximately 12 days. It is easy to see how one could be misled into thinking equilibrium had been obtained if the solubility were determined solely at several hourly intervals. Thus, it is of importance to adequately define the physical as well as the chemical nature of the substance under study, before and after the solubility determination, so that the thermodynamic parameters can be assigned to the proper species. Knowledge of the physical state allows for meaningful expression of equilibrium solubility.

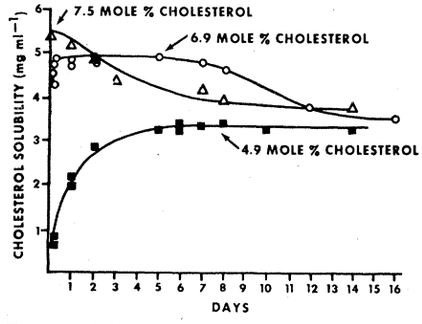

Fig. 1. Dissolution behavior of several forms of cholesterol in lecithin–bile salt system; coprecipitated (triangles), anhydrous (circles), and monohydrated (squares) cholesterol. The inserted mole percentages express the maximal solubility obtained with each form of cholesterol.

The equilibrium solubility of cholesterol in these model systems, designed to mimic the biliary lipid milieu, is in the order of 5 mole percent [unit defined in (*7*)]. This may be of medical significance, because the cholesterol concentrations in the bile of normal humans (no gallstones) have been reported to be far greater than 5 mole percent (*7, 8*). This would imply the presence of supersaturation in many normal biles and raise questions about the factors responsible for the maintenance of the supersaturation. The potential for cholelithiasis may be far greater than is presently imagined. The "forgotten," water-soluble components of bile, which contribute little to the solubilization of cholesterol, may be the determinants of whether or not cholesterol precipitates from supersaturated media.

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Acrosomal Proteinase and Proteinase Inhibitor of Human Spermatozoa

Abstract. *The acrosomal proteinase of human spermatozoa was characterized and differs from other human proteinases. The enzyme has optimal activity at* p*H 8.0, is inactive below* p*H 5.0 or above* p*H 10.5, requires calcium for maximum activity, hydrolyzes fibrinogen, gelatin, and benzoylarginine ethyl ester, and is inhibited by various synthetic and natural proteinase inhibitors. The molecular weight was estimated to be 30,000. Human spermatozoa also possess a proteinase inhibitor that is similar to one of the proteinase inhibitors from human seminal plasma.*

Recently the proteinase of spermatozoa has received considerable attention. It was shown to be present in all mammalian sperm studied including those of the rabbit (*1–6*), rat (*5, 6*), guinea pig (*5*), mouse (*5, 6*), hamster (*6*), stallion (*5*), boar (*5, 7*), ram (*1, 5*), bull (*1, 5*), human (*3, 5, 6*), and rhesus (*3*) and squirrel monkeys (*5*). Gelatin slide experiments using whole sperm cells (*6*) substantiated previous evidence (*1, 2*) that the proteinase is located in the acrosome of the spermatozoon. Sperm acrosomal preparations dissolve the zona pellucida of rabbit ova (*1, 2*), a process that can be inhibited by proteinase inhibitors (*2*). Fertilization of rabbit ova is prevented in vitro by addition of soybean trypsin inhibitor to the sperm-egg medium (*8*) and in vivo if capacitated sperm—that is, sperm that resided in the female genital tract for at least 6 hours—are first incubated with the pancreatic trypsin inhibitor (Kunitz) or inhibitors isolated from rabbit seminal plasma before insemination into the oviducts of donor rabbits (*9*). Tosyllysine chloromethyl ketone (TLCK), a synthetic trypsin inhibitor, prevents fertilization when added to the rabbit vagina before coitus (*10*).

These experiments emphasize the important role of the sperm acrosomal proteinase in the reproductive process and show that the inhibition of this enzyme prevents fertilization. Although the rabbit sperm acrosomal proteinase has been studied (*11, 12*), no data are available concerning the acrosomal proteinase of human spermatozoa. The properties of this enzyme were determined, with special emphasis on the inhibitory effect of natural and synthetic proteinase inhibitors. During the initial study it became apparent that the acrosomal extracts of human spermatozoa contain an inhibitor of the acrosomal proteinase. The properties of this inhibitor were also evaluated.

Freshly collected human semen samples were centrifuged to remove the seminal plasma and washed three times with 0.15*M* NaCl. Acrosomal extracts were prepared by incubating the spermatozoa with 0.075 percent Hyamine 2389 (Rohm and Haas) and 0.075 percent Triton X-100 (Rohm and Haas) for 90 minutes at 37°C (*7*). The suspension was centrifuged, the supernatant solution was treated with ethanol (80 percent), and the precipitated protein was extensively dialyzed and lyophilized. The acrosomal preparations were tested for activity by dissolving in 0.1*M* borate buffer, *p*H 8.0, containing 0.05*M* $CaCl_2$ and subsequent addition to benzoylarginine ethyl ester (BAEE, 0.2 mg/ml) as substrate in the same buffer (*13*). One milliunit (mU) was defined as that amount of enzyme that caused a change in absorbance of 0.001 per minute at a wavelength of 253 nm. After Sephadex-gel filtration, acrosomal inhibitor activity was determined by incubating 0.1 ml of the inhibitor solution, adjusted to *p*H 8.0, with 0.1 ml bovine pancreatic trypsin (Worthington) (3 μg) for 5 minutes at room temperature before

Table 1. Comparative properties of various human proteinases. Inhibitor tests were made by incubating 0.1 ml of inhibitor in saline with 0.1 ml of the acrosomal proteinase in 0.1*M* borate buffer, *p*H 8.0, containing 0.05*M* $CaCl_2$, for 5 minutes at room temperature before addition of 0.1 ml of the mixture to BAEE. As control, the acrosomal proteinase was incubated with saline. Inhibition is shown as +, no inhibition as −. Inhibition is marked as progressive or immediate for those enzymes and inhibitors for which this has been determined.

Properties	Acrosomal proteinase	Pancreatic trypsin	Plasmin	Thrombin	Reference
Molecular weight	30,000	22,900	75,400	31,000	(*18, 19*)
Effect of inhibitors					
Tosyllysine chloromethyl ketone	+	+	+	+	(*18, 20, 21*)
Tosylamide phenylethyl chloromethyl ketone	−	−	−	−	(*18, 20, 21*)
Diisopropyl fluorophosphate	+	+	+	+	(*18, 21–23*)
Soybean trypsin inhibitor	+	+ (weak)	+	−	(*18, 24*)
Trypsin-kallikrein inhibitor (Kunitz)	Progressive	Immediate	+	−	(*18, 24*)
Potato trypsin inhibitor	+	−	−	−	(*24*)
Ovomucoid trypsin inhibitor	+ (weak)	−	−	−	(*24*)
Inter-α-trypsin inhibitor	+	+*	−	−*	(*25*)
α_1-Antitrypsin	Progressive	Immediate*	Progressive	−*	(*25*)
Antithrombin III	+	+*	+ (weak)	+*	(*25*)
α_{1x}-Antichymotrypsin	−	−*	−	−*	(*25*)

* Results are available for species other than the human only.