Solubilization of unconjugated bilirubin by bile salts

U. Wosiewitz and S. Schroebler

Institut für Medizinische Physik der Universität Münster, Hüfferstr. 68, D-4400 Münster (Federal Republic of Germany), 17 October 1978

Summary. Freshly precipitated unconjugated bilirubin (UCB) is solubilized rapidly and to a large extent by the sodium salts of di- and trihydroxy bile acids. The solubilization effect depending on bile salt concentration, pH and ionic strength is based on micellar mechanisms.

Unconjugated bilirubin (UCB) is poorly soluble in water or in buffer solutions with a pH below the p_k of UCB (7.95)[1,2]. On the other hand, UCB concentration in the bulk of gallbladder bile (pH 6.8–7.4) is much greater than that calculated from the p_k. This indicates that some bile constituents (i.e. bile salts) cause the increase in UCB solubility. Solubilization of UCB by bile salts has been studied by only a few authors[3–5] and the mechanism of solubilization is still under discussion.

In order to study the effect of bile salts, bile salt-lecithin and bile salt-lecithin-cholesterol mixed micelles on the solubility of UCB, we used both pure dry UCB and – in contrast to other investigators – freshly precipitated UCB.

Methods. 1. Solubilization of solid, dry UCB. An excess of dry UCB (analytical grade from Serva) was suspended in buffered solutions of sodium taurocholate (Na-TC), sodium taurodeoxy cholate (Na-TDC), sodium taurochenodeoxy cholate (Na-TCDC) and sodium dehydro cholate (Na-DHC). All bile salts were from Sigma and were used without further purification. Bile salt concentrations reached from 5 to 150 mM. Solubilization was studied at pH 7.5 in 0.2 M phosphate buffer. UCB suspensions were given to brown 10-ml glass flasks, sealed under N_2 and shaken for 3 days at 37 °C. The suspensions were aspirated with a syringe and passed through a 0.2-µm millipore filter. Filtrate concentrations of UCB were quantified immediately by photometric assay of azobilirubin according to the method of Jendrassik and Gròf[6], using Boehringers 'Test-combination' for bilirubin (Boehringer Diagnostica, Mannheim).

2. Solubilization of freshly precipitated UCB. UCB was dissolved in 0.2 M NaOH (cooled at 4 °C, saturated with N_2). The true solution of sodium bilirubinate was stirred under N_2 and adjusted to a required pH (6–8) by adding an appropriate volume of a cold, N_2-saturated 0.2 M KH_2PO_4 solution. The final UCB concentrations of the prepared suspensions were 1000–1200 mg/dl (17.1–20.5 mM). Bile salts were added to each 2 ml of the suspension giving bile salt concentrations of 5–80 mM. The suspensions were incubated at 37 °C for 4–6 h, then filtrated as described above and subjected to UCB determination (s.a.). The solubilization effect of mixed micelles was studied in the same way by preparing mixtures of Na-TC/lecithin (dipalmitoyl phosphatidylcholin, analytical grade from Sigma) with molar ratios of 6:1 and 3:1, respectively, and of Na-TC/lecithin/cholesterol (cholesterol isolated from gallstones and purified by column chromatography) with a molar ratio of 6:1:1 (for preparation of mixed micellar solutions see Paul et al.[7]). The effect of ionic strength on the solubilization of UCB by Na-TC was studied by adding different amounts of NaCl to the test solutions to give Na^+-concentrations from 0.1–0.5 M.

Results. The solubilization effect of bile salts on solid pure UCB was found to depend on bile salt concentration but altogether it was very small. Maximum UCB filtrate concentrations never exceeded $6 \cdot 10^{-5}$ M. By contrast, freshly precipitated UCB was dissolved rapidly and to a very large extent by all the bile salts used exept Na-DHC (figure 1). Solubilization by pure buffer solutions at $pH \leq 7.5$ was negligible (UCB filtrate concentrations $< 1.7 \cdot 10^{-6}$ M), but became increased at pH > 7.5 (at pH $8 \approx 2.5 \cdot 10^{-4}$ M).

The effect of pH on UCB solubilization is demonstrated in figure 2. Maximum solubilization was achieved at pH 7.5, but even at pH 6, where nearly all UCB is protonated, solubilization is considerable. Effect of ionic strength: UCB filtrate concentration became slightly decreased when the molarity of added NaCl was increased. Effect of mixed micelles: Na-TC/lecithin mixtures with molar ratios of 6:1 or 3:1 showed approximately the same solubilization effect as was observed with pure Na-TC solutions having a corresponding molarity. By contrast, using Na-TC/lecithin/cholesterol mixed micellar solutions with a molar ratio of 6:1:1, solubilization of UCB was about 30% lower

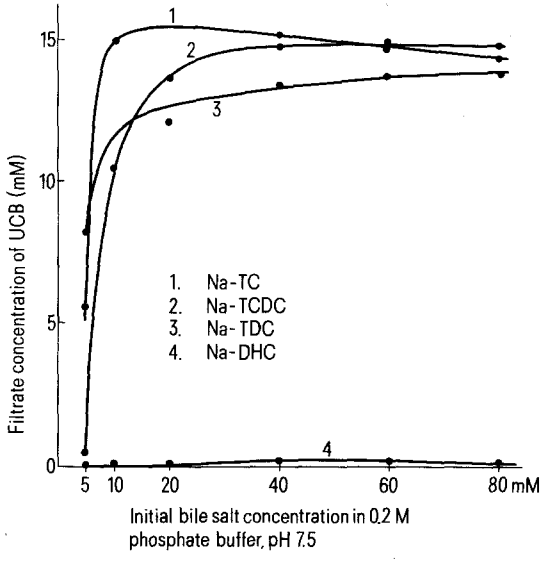


Fig. 1. Solubilization of unconjugated bilirubin (UCB) by bile salts in 0.2 M phosphate buffer, pH 7.5, t = 37 °C. UCB filtrate concentrations diminished by UCB solubility in pure phosphate buffer (mean values from each 5 solubilization experiments).

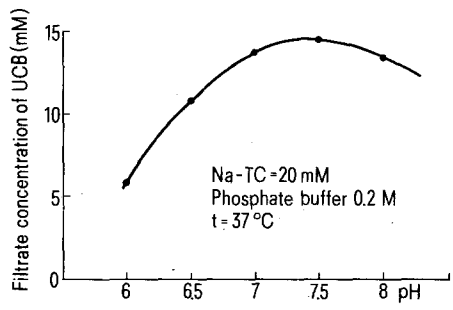


Fig. 2. pH-dependence of UCB solubilization by Na-TC. UCB filtrate concentrations diminished by UCB solubility in pure phosphate buffer (mean values from each 5 solubilization experiments).

compared with Na-TC or Na-TC/lecithin solutions. UCB oxidation: Some of the solubilized UCB became irreversibly oxidized during incubation. Transformation to biliverdin was checked by VIS-spectroscopy, which showed that less than 2% of UCB was oxidized to biliverdin during the whole experimental procedure. Standard deviations: Standard deviation of photometric UCB assay was calculated to be better than ±2%. Within a series of each 5 solubilization experiments, using the same bile salt concentration, standard deviation was about ±6.5% (with respect to UCB filtrate concentration).

Discussion and conclusion. The most striking result is the considerable solubilization effect of di- and trihydroxy bile salts on freshly precipitated but not commercial solid UCB. We used the precipitating procedure to obtain UCB in a physical state closely similar to the one in bile.

From the results we conclude that UCB solubilization by bile salts is based on micellar mechanisms, i.e. UCB is considered to enter the bulk of the bile salt micelle. Consequently, the solubilization effect of Na-DHC which does not form micelles, is expected to be small. In fact this was observed (figure 1). Furthermore, Na-TC/lecithin micelles which already have absorbed cholesterol, are somewhat prevented from solubilizing UCB (see above).

In bile, several lipids, especially cholesterol, compete for the mixed bile salt/phospholipid micelles. We postulate that a micelle which is saturated with cholesterol, will poorly solubilize UCB in bile. Thus, solubilization of UCB in bile very much depends on the degree of cholesterol saturation of mixed micelles. Biles where UCB is increased either by an elevated biliary excretion and/or by increased hydrolysis of conjugated bilirubin, will consequently become supersaturated with UCB if the solubilizing power of mixed micelles is mainly claimed by cholesterol and other insoluble lipids.

1 J.T.G. Overbeeck, C.L.J. Vink and H. Deenstra, Recl Trav. chim. *74*, 81 (1955).
2 R.C. Burnstine and R. Schmid, Proc. Soc. exp. Biol. Med. *109*, 356 (1962).
3 T.J. Devers, D. Gallo and J.D. Ostrow, Gastroenterology *69*, 816 (1975).
4 T.J. Devers, D. Gallo and J.D. Ostrow, in: The Liver. Quantitative Aspects of Structure and Function, p.429. Aulendorf 1976.
5 S. Yamamoto, Acta med. (Fukuoka) *28*, 28 (1958).
6 L. Jendrassik and P. Gròf, Biochem. Z. *297*, 81 (1938).
7 R. Paul and P. Balaram, Biochem. biophys. Res. Commun. *81*, 850 (1968).

Peroxidase activity in the sponge, *Iotrochota birotulata*[1]

M.D. Corbett and Bernadette R. Chipko

Rosenstiel School of Marine and Atmospheric Science, University of Miami, 4600 Rickenbacker Causeway, Miami (Florida 33149, USA), 4 September 1978

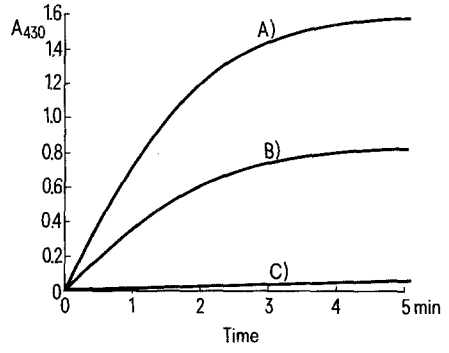
Summary. The sponge *Iotrochota birotulata* contains a peroxidase which was partially characterized. This is the first report of a peroxidase in Porifera, originally thought to be devoid of such enzymatic activity.

Sponges have proven to be a rich source of novel natural products, including brominated compounds[2] and compounds with interesting oxygenation patterns[3]. No attempts have yet been made to characterize those enzymes that are responsible for the production of such natural products, although it has been suggested that brominated marine natural products are the result of peroxidative enzymes similar to the well known terrestrial fungal enzyme, chloroperoxidase[4]. The only reported attempt to detect peroxidase activity in sponges concluded that peroxidases are absent in this phylum[5]. Unfortunately, this conclusion was based upon the investigation of only three genera of porifera. We now wish to report on the strong peroxidatic activity in a marine sponge, and to correct the phylogenetic conclusion that primitive animals, such as the sponges, lack this activity.

Our interest in invertebrate peroxidases arose from our finding that chloroperoxidase oxidizes arylamines to arylnitroso compounds[6]. The possible environmental consequences of such transformations were also recently proposed[7].

Our attention was drawn to *Iotrochota birotulata* (Higgins) which produces a dark purple colored, high mol. wt pigment when damaged, hence, its common name 'purple bleeding sponge'[8].

Materials and methods. Specimens were collected on 3 separate occasions in about 6 m of water on a coral reef 10 km southeast of Key Biscayne and returned immediately to the laboratory for processing. Identification of the species was made by us according to Wiedenmayer[9] and confirmed by J. Garcia-Gomez (University of Miami). Careful examination of the surfaces and of longitudinal sections of specimens indicated the absence of contaminating macroorganisms, such as commonly found in larger sponge species. In fact, we suspect that this sponge might contain toxic components, as we have never observed other benthic invertebrates or macroalgae growing in close asso-



Oxidation of pyrogallol by *I. birotulata* peroxidase. To a 1-cm quartz cuvette containing 20 µmoles of pyrogallol, 12 µmoles of H_2O_2 and 100 µmoles of potassium phosphate buffer, pH 6.0, in 2.0 ml of H_2O was added, to initiate the reaction: A) 0.20 ml of enzyme preparation; B) 0.10 ml of enzyme preparation and C) 0.10 ml of enzyme preparation that had been heated for 5 min at 80 °C.