Bilirubin/Rat Serum Albumin Interaction

Peder C. Frandsen and Rolf Brodersen*

Institute of Medical Biochemistry, University of Aarhus, DK-8000 Aarhus C, Denmark

Frandsen, Peder C. and Brodersen, Rolf, 1986. Bilirubin/Rat Serum Albumin Interaction. – Acta Chem. Scand. B 40: 55–59.

Essential differences are demonstrated between bilirubin binding to rat serum proteins and to albumin in human serum. Acidimetric titration of rat serum with and without added bilirubin shows that binding of bilirubin acid in the range of pH from 6.8 to 8.8 takes place with release of less than one hydrogen ion per molecule of bound bilirubin. With human serum, two hydrogen ions are released, indicating binding of bilirubin dianion. The binding equilibrium of N-[4-[(4-aminophenyl)-sulfonyl]phenyl]-acetamide (MADDS) to rat serum albumin is influenced slightly by cobinding of bilirubin whereas MADDS and bilirubin bind competitively to human serum albumin. Finally, the rate of oxidation of bilirubin with hydrogen peroxide and peroxidase is decreased moderately by addition of rat serum albumin and strongly by the human protein, indicating that biliribin in its complex with rat serum albumin is subject to oxidation while the complex with human serum albumin is protected. These differences should be considered when rats are used as a model in experimental studies aiming at prevention of bilirubin encephalopathy in human neonates.

Bilirubin, a dicarboxylic acid, circulates in human blood plasma where it is bound to serum albumin. In the complex with human albumin, bilirubin is present as the dianion and the binding affinity is independent of pH in a range from 7 to 9.[1] If a sulfonamide, which binds compettitively with bilirubin, is added to the plasma, or if pH is decreased, conditions become favourable for precipitation of bilirubin acid which is slightly soluble in water. The solubility of bilirubin acid in water at 37 °C is about 10^{-15} M.[2] The first stoichiometric binding constant for the bilirubin dianion to human serum albumin at 37 °C at 0.15 M ionic strength is 6×10^{7} M^{-1}.[3] The mean pK_a for the two carboxyl groups is 4.4.[4] From these data it is possible to calculate when conditions favour precipitation of bilirubin acid and when dissolution of the precipitated acid can take place. Quantitative agreement of these calculations with clinical observations of bilirubin deposition in the brain of newborn infants after giving a sulfonamide[5] or in acidotic newborns,[6,7] and the fact that such precipitation does not take place in the healthy newborn with a normal plasma pH[7] have suggested that yellow staining of certain areas of the brain (kernicterus) in human infants can be explained by simple precipitation of bilirubin acid.[2]

What appears to be a different process, transfer of the entire bilirubin-albumin complex into the brain, has been observed in rats after disruption of the blood-brain barrier by hyperosmosis[8] or by increased carbon dioxide tension.[9] The possible role of this mechanism in human kernicterus is difficult to evaluate at present.

Rats have further been used in experiments with bilirubin transfer from blood to brain, induced by sulfonamides[10,11] and it has been suggested that screening of drugs for bilirubin displacing properties could be undertaken in rats.[12,13] Measurement of free bilirubin concentration in blood plasma of rats has further been attempted, using peroxidase rate determination. Such determinations are made on the presump-

*To whom correspondence should be addressed.

tion that bound bilirubin is protected from oxidation; the rate of the peroxidase process is considered proportional to the free equilibrium concentration of bilirubin dianion.[10,14]

In all these cases it is important to know whether the bilirubin dianion is bound to rat albumin as it is to the human protein, whether bilirubin competes with binding of the same substances, and whether bilirubin is protected from peroxidase oxidation when bound to rat albumin, as it is in the complex with human protein. We undertook a few experiments to elucidate this question.

Materials and Methods

Human serum albumin was obtained from AB KABI, Stockholm, Sweden, and was defatted with charcoal in acid solution.[15] Rat serum was obtained from Wistar rats. Rat serum albumin was prepared by fractionating rat serum on a Sephacryl S-300 column using a sodium borate buffer pH 8.4 and then dialyzing against water before freeze drying. Bilirubin was from Sigma, Saint Louis, USA, and was purified as described by McDonagh and Assisi.[16]

Acidimetric titrations were carried out at 25.0 °C in a carbon dioxide free atmosphere, using a glass electrode No. G-2040B and a reference calomel cell No. K102-K connected to a salt bridge No. K102-B from Radiometer, Copenhagen, Denmark. The reference electrode as well as the titration vessel were kept at 25 °C during a period of at least 24 h prior to and during titration. Readings of pH were made to three digits after the decimal point. Low-carbonate sodium hydroxide solution was prepared by dissolving 100 g of sodium hydroxide in 100 ml of warm water and leaving the solution for precipitation of sodium carbonate. Titration was carried out with 2 M hydrochloric acid from an "Agla" micrometer syringe from Wellcome Reagents Ltd., Dartford, England, allowing a definition of a 500 µl volume to 0.02 µl. The burette barrel was protected from temperature fluctuations and the entire titration setup was confined to a soft-iron plate cabinet to prevent electric noise.

Bilirubin, 2.0 µmol, was dissolved in a measured surplus of sodium hydroxide solution, *rat* or *human* serum was added to obtain an albumin concentration approximately equal to that of bilirubin, 100 µM, and acidimetric titration was carried out with 2 M hydrochloric acid. The experiments were repeated without addition of bilirubin. The difference in the amounts of hydrochloric acid used in two experiments with and without bilirubin, divided by the amount of bilirubin, was then plotted as a function of pH.

Binding of MADDS was studied by a previously described microdialysis technique.[17] A small amount of ^{14}C-MADDS†, final concentration 3 µM, is added to a buffered albumin solution with pH 7.4 and dialyzed against an identical solution without MADDS. The radioactivity is measured on both sides of the membrane after 10 min dialysis at 37.0 °C. Equilibrium between the two compartments is not established during this time and the rate of dialysis is taken as a measure of the free concentration of MADDS in binding equilibrium with albumin.

The rate of oxidation of bilirubin in buffered albumin solutions with hydrogen peroxide and horse radish peroxidase was studied as previously described.[5] All experiments were performed at 37.0 °C in a 60 mM sodium phosphate buffer, pH 7.4.

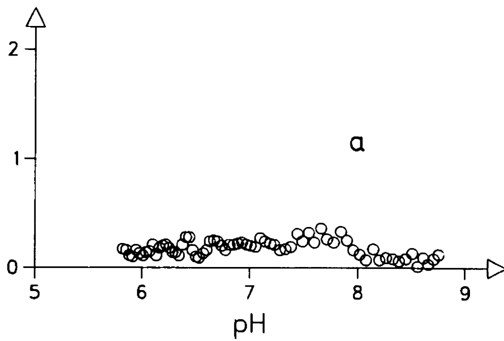
Results

In Fig. 1a the number of acid protons dissociated from bilirubin when present in *rat* serum is plotted as a function of pH. It is noted that bilirubin, in an interval of pH from 6.8 to 8.8, dissociates less than one proton. In each of two experiments the average throughout this range of pH was 0.2 and 0.6 H^+ dissociated from each bilirubin molecule. Bilirubin was thus present predominantly as the acid.

The results using *human* serum, are pictured in Fig. 1b. The average number of dissociated protons was in this case 1.7 and 1.9 in two experiments, when measured in the pH interval from 6.8 to 8.8. Bilirubin was present predominantly as the dianion. When titration was continued to lower pH, bilirubin was partially converted to the acid.

Fig. 2 indicates that MADDS, added to rat serum, is bound with rather low affinity, when compared to the affinity for human surum. Addition of bilirubin causes little change of MADDS binding to rat serum whereas a considerable de-

† Abbreviation: MADDS, *N*-[4-[(4-aminophenyl)-sulfonyl]phenyl]-acetamide.



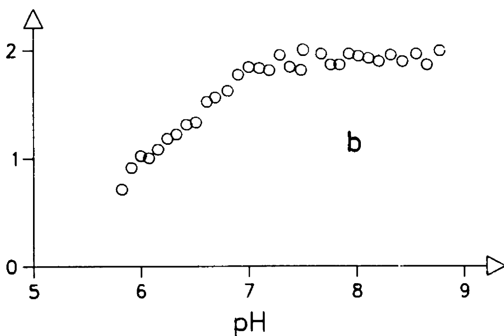


Fig. 1. Acidimetric titration was made on serum diluted to approximately 100 μM albumin, with and without added bilirubin acid, 100 μM. The ordinate is the difference of amounts of hydrochloric acid used in the two experiments, divided by the amount of bilirubin, a) with rat serum, b) with human serum.

crease of MADDS binding is seen when bilirubin is added to human serum.

Further experiments with purified rat and human serum albumins (isolated as described for rat serum albumin in Methods) have shown that the differences of MADDS binding affinity, illustrated in Fig. 2, are due to different affinities of MADDS for the two proteins. It has further been confirmed that MADDS-bilirubin competition fails with the purified rat protein. Defatting as described by Chen[15] of both protein species does not alter these results.

It has previously been found that one molecule of MADDS binds to human serum albumin, competing with one molecule of bilirubin, and this has been utilized in a practical method for estimating the bilirubin binding properties of infant sera.[17] The MADDS method obviously cannot be used for this purpose in rat experiments, due to a difference of binding mechanisms.

Fig. 3 illustrates that rat serum albumin has a limited effect on the rate of peroxidase oxidation of bilirubin while a marked inhibition is seen with the human protein. Oxidaton rates of bilirubin in a buffered solution with a two- to eight-fold molar surplus of albumin were measured. Initial oxidation rates were plotted as a function of the reciprocal of the albumin minus bilirubin concentrations. Under these circumstances the free equilibrium concentration of bilirubin is approx-

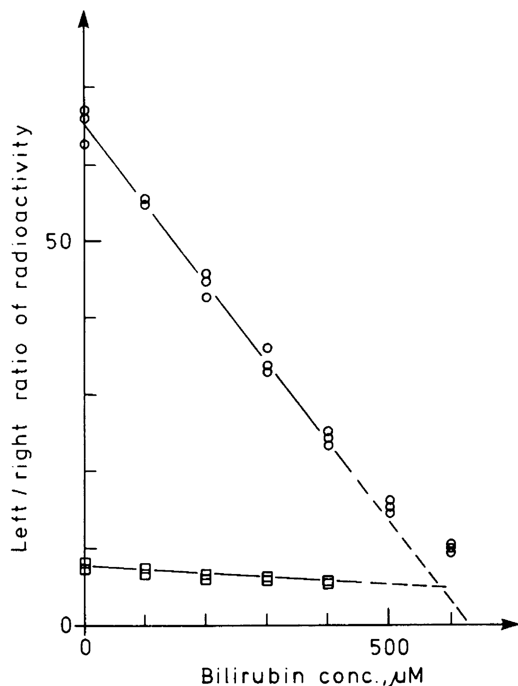


Fig. 2. Non-equilibrium dialysis of ^{14}C-MADDS, 3 μM, added to serum on the left side of a cellophane. An identical serum sample was present on the right side. Ratios of radioactivity, left/right side, were measured after 10 min of dialysis (ordinates). Varying concentrations of bilirubin (abscissa) were added on both sides of the membrane. Experiments with human serum, ○, indicate tight binding of MADDS and marked competition with binding of bilirubin. In rat serum, □, binding of MADDS occurs with lower affinity, as indicated by faster dialysis, and is less influenced by biliburin.

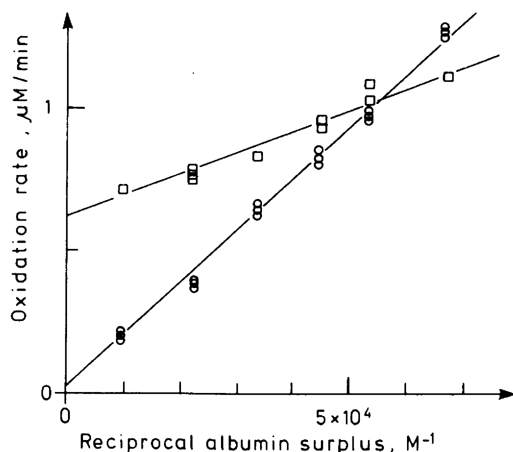


Fig. 3. Oxidation rates (ordinates) for bilirubin with hydrogen peroxide and peroxidase, measured with varying molar surplus of serum albumin, ○, human serum albumin; □, rat serum albumin. The peroxidase concentration was 24 nM in experiments with the human protein, and 6 nM with rat serum albumin. The bilirubin concentration was constant, 15 μM, throughout.

imately inversely proportional to the albumin surplus. A straight line through the origin is thus expected if the rate of oxidation is proportional to the free bilirubin concentration. A positive intercept with the ordinate axis shows the rate of oxidation of bound bilirubin. Fig. 3 illustrates that the oxidation rate for bilirubin bound to human serum albumin is negligible; only the free bilirubin is oxidized. With rat serum albumin, the rate of oxidation of bound bilirubin is considerable. In a solution containing 15 μM bilirubin and 30 μM rat serum albumin, corresponding to the extreme right of the graph, more than half of the oxidation concerns bound bilirubin. At 400 μM, albumin, as found in rat serum, with low bilirubin concentrations, practically only bound bilirubin would be oxidized.

Discussion

It has previously been shown that one molecule of bilirubin in neutral and slightly alkaline solutions is bound to human serum albumin as a dianion.[3] The chromophores of the bound dianion are probably internalized in the albumin molecule.[18] The fact that bound bilirubin is resistant to oxidation with peroxidase may be due to internalization. The presence of two negatively charged carboxylate groups in the bound molecule may also contribute to a low oxidation rate. It is difficult to evaluate the latter effect since the carboxylate groups may be located at a distance from the aromatic chromophores where oxidation takes place. Peroxidase oxidation of bilirubin, bound to rat serum albumin, proceeds much faster, as shown in the present paper. This may be explained alternatively by lack of internalization or by the absence of negatively charged carboxylate groups.

A buffered solution of human serum albumin and bilirubin will contain free bilirubin dianion in a certain equilibrium concentration and further a very small amount of bilirubin acid with both carboxyl groups in the protonated form. If the equilibrium concentration of bilirubin acid is larger than the solubility, precipitation of bilirubin acid is thermodynamically possible; precipitation may take place, or the solution may remain in a supersaturated state. Supersaturation is often seen in actual experiments *in vitro*.

When precipitation of bilirubin acid takes place in the presence of a phospholipid membrane, as *in vivo* where blood plasma is in intimate contact with cell surfaces, the process occurs promptly and proceeds with formation of bilirubin acid aggregates which have a somewhat higher solubility than crystalline bilirubin acid.[19] It seems possible to explain deposition of bilirubin in the brain of newborn infants as a result of this process.[20] It is then theoretically expected that the deposition can be induced by occupation of albumin in blood plasma by a competitively bound sulfonamide. Transfer of bilirubin to the brain would also be expected to occur at lower plasma bilirubin concentrations if the binding affinity of the albumin is reduced. We would finally expect that a low plasma pH would increase the risk of bilirubin deposition. All three expectations are fulfilled by clinical experience; giving sulfonamides may precipitate kernicterus;[21] infants with reduced bilirubin binding properties of serum albumin, as seen in respiratory distress syndrome or in severe infections, constitute a particular risk group showing signs of bilirubin damage at lower plasma bilirubin concentrations than other infants;[6,22] and acidosis increases the risk.[23]

A theory of simple bilirubin acid precipitation

is thus supported qualitatively by these observations and is further substantiated by calculations based upon the solubility, the acid dissociation constants of the two carboxyls, and the binding affinity of bilirubin to albumin, indicating that precipitation of bilirubin does in fact take place in cases where it is theoretically expected.[7] The above theory has been developed on the basis of *in vitro* measurements and clinical observations in human neonates. In rat experiments it has been found that sulfonamides displace bilirubin from blood to brain;[10,11,24] however, acidosis does not promote bilirubin deposition,[9] attempted measurements of free bilirubin concentrations by means of the peroxidase technique do not give the expected results,[9] and a different mechanism of bilirubin transfer, involving the entire bilirubin-albumin complex, has been found.[8,9] These observations are understood when it is considered that binding of bilirubin to rat serum albumin occurs by a different mechanism, deviating from the simple binding of bilirubin dianion to human albumin.

It should further be noted that the binding affinity for bilirubin to rat serum albumin remains unknown. Generally, binding of drugs to rat serum albumin has not been studied in detail and the present findings illustrate that binding competition of drugs with bilirubin may follow different patterns from those seen with the human protein. It is consequently not possible to use the rat as an animal model in studies of drug or acidosis induced bilirubin encephalopathy of the human neonate.

Acknowledgements. The authors wish to express their thanks to Signe Andersen, Nina Jørgensen and Birthe Lindgaard for technical work, and to Frede Nielsen for computer and graphical assistance. A desk computer, HP-85 from Hewlett Packard, was obtained from the Danish Medical Research Council through grant No. 12-2365.

References

1. Nelson, R., Jacobsen, J. and Wennberg, R. P. *Pediatr. Res. 8* (1974) 963.
2. Brodersen, R. *CRC Crit. Rev. Clin. Lab. Sci. 11* (1979) 305.
3. Brodersen, R. *J. Biol. Chem. 254* (1979) 2364.
4. Hansen, P.E., Thiessen, H. and Brodersen, R. *Acta Chem. Scand. 33* (1979) 281.
5. Brodersen, R. *J. Clin. Invest. 54* (1974) 1353.
6. Ebbesen, F. and Brodersen, R. *Early Human Development 6* (1982) 341.
7. Cashore, W. J., Oh, W. and Brodersen, R. *Acta Paediatr. Scand. 72* (1983) 415.
8. Levine, R. L., Fredericks, W. R. and Rapoport, S. I. *Pediatrics 69* (1982) 255.
9. Bratlid, D., Cashore, W. J. and Oh, W. *Pediatrics 73* (1984) 431.
10. Øie, S. and Levy, G. *J. Pharm. Sci. 68* (1979) 6.
11. Bratlid, D., Cashore, W. J., Brubakk, A. M. and Oh, W. *Pediatr. Res. 18* (1984) 150A.
12. Nathenson, G., Cohen, M. I. and McNamara, H. *J. Pediatr. 86* (1975) 799.
13. Ballowitz, L. *J. Pediatr. 92* (1978) 166.
14. Jacobsen, J. and Wennberg, R. P. *Clin. Chem. 20* (1974) 78.
15. Chen, R. F. *J. Biol. Chem. 242* (1967) 173.
16. McDonagh, A. F. and Assisi, F. *Biochem. J. 129* (1972) 797.
17. Brodersen, R., Andersen, S., Jacobsen, C., Sønderskov, O., Ebbesen, F., Cashore, W. J. and Larsen, S. *Anal. Biochem. 121* (1982) 395.
18. Jacobsen, J. and Brodersen, R. *J. Biol. Chem. 258* (1983) 6319.
19. Eriksen, E. F., Danielsen, H. and Brodersen, R. *J. Biol. Chem. 256* (1981) 4269.
20. Brodersen, R. *Bilirubin, Vol. 1, CRC Press* (1982) 75.
21. Silvermann, W. A., Andersen, D. H., Blanc, W. A. and Crozier, D. N. *Pediatrics 18* (1956) 614.
22. Gartner, L. M., Snyder, R. N., Chabon, R. S. and Bernstein, J. *Pediatrics 45* (1970) 906.
23. Stern, L. and Denton, R. L. *Pediatrics 35* (1965) 483.
24. Johnson, L., Sarmiento, F., Blanc, W. A. and Day, R. *Am. J. Dis. Child. 97* (1959) 591.

Received May 5, 1985.