The Interaction of Calcium Ions with Glycocholate Micelles in Aqueous Solution

By Barry W. A. WILLIAMSON and Iain W. PERCY-ROBB
*University Departments of Clinical Surgery and Clinical Chemistry,
The Royal Infirmary, Edinburgh EH3 9 YW, Scotland, U.K.*

(Received 4 December 1978)

The formation of soluble complexes of Ca^{2+} ions and glycocholate has been demonstrated. The dissociation constant is 26 mmol/litre and a maximum of 2 Ca^{2+} ions are bound to each glycocholate micelle. The formation of this complex is shown to be reversible. Binding is increased by the introduction of phosphatidylcholine into the micelle; it is decreased by a decrease in pH and by increased counter-ion concentration. The biological significance of these effects is discussed.

Cholesterol, both in its monohydrate and anhydrous forms, and insoluble salts of Ca^{2+} are the predominant components of gallstones obtained from patients in Western Europe (Sutor & Wooley, 1973). The Ca^{2+} salts show a variety of physical forms including vaterite (CaCO_3) and apatite (calcium phosphate).

Little is known about factors that lead to the formation of gallstones. Failure of the bile-salt and phospholipid system in bile to retain cholesterol in solution may be one important factor (Admirand & Small, 1968). However, whereas cholesterol microcrystals form *in vitro* in bile salt/cholesterol/phospholipid/water mixtures, the theory that these microcrystals act as a nidus for gallstone formation does not explain the presence of Ca^{2+} salts in gallstones. Furthermore, bile obtained from patients who do not have gallstones is supersaturated with respect to cholesterol for several hours each day (Northfield & Hofmann, 1975), and yet it has been estimated that only about 10% of the population in Scotland and England have gallstone disease (Watkinson, 1967).

It is possible that microcrystals of insoluble Ca^{2+} salts act as a nidus on which both additional Ca^{2+} and cholesterol are subsequently deposited, thus forming a gallstone. The potential for formation of Ca^{2+} microcrystals is related to the activity of ionized calcium rather than to its total concentration in bile. It is thus important to determine which of the various molecular species in bile bind Ca^{2+} , thereby decreasing its activity, and to examine factors that affect binding.

In the present paper we describe experiments in which we study (1) the binding of Ca^{2+} to glycocholate, the predominant bile salt in human bile (Hofmann & Small, 1967), (2) the effects of variations

in pH and ionic strength and (3) the effect of adding phosphatidylcholine to the system.

Materials and Methods

Ca^{2+} binding

Binding of Ca^{2+} to glycocholate was studied by ultrafiltration and by direct activity measurement by using a calcium electrode.

Ultrafiltration was carried out at 37°C in a 13 mm cell (Millipore U.K., London, U.K.) fitted with a membrane (PSAC 01310) having a molecular-weight cut-off of 1000. The ultrafiltration experiments were performed with sodium glycocholate solutions in Tris/HCl buffer [Tris/HCl (20 mm); NaCl (150 mm); pH 8.00]. To examine the reversibility of binding, a reservoir was fitted to the cell to ensure that the studies were performed at constant volume. Filtration was performed under an N_2 pressure of 170 kPa. The retention characteristics of the membrane and the performance of the constant-volume cell have been described (Williamson *et al.*, 1978). These constant-volume experiments were performed at 22°C.

Solutions of sodium glycocholate (40 mm) in Tris/HCl buffer, pH 8.00 and pH 7.20, were ultrafiltered. The mean concentration of glycocholate in the filtrate was 4.1 mm (range 3–5 mm). Since the critical micellar concentration of glycocholate is in the range 3–6 mm (Small, 1971) we concluded that micelles were retained by the ultrafiltration membrane at both pH values. The retention of glycocholate by the ultrafiltration membrane was not affected by addition of NaCl (0–250 mm, pH 8.00). CaCl_2 (0.1, 0.5, 5.0, and 9.0 mm) in Tris/HCl was ultrafiltered and the recovery of Ca^{2+} in the filtrate was 99.1–99.9%.

The range of total Ca^{2+} concentrations used in the binding studies was 1–12 mM, which is similar to the total Ca^{2+} concentrations found in human bile (Nakayama & Van der Linden, 1970). In the binding studies, Ca^{2+} was added to the sodium glycocholate solutions as the chloride containing $^{45}\text{CaCl}_2$. Radioactivity in individual drops of ultrafiltrate (20 μl) was measured by liquid-scintillation counting (Strange *et al.*, 1976). The concentration of Ca^{2+} in each sample of ultrafiltrate was calculated from the measured radioactivity and from the specific radioactivity of the CaCl_2 added to the sodium glycocholate solution. The Ca^{2+} that could be ultrafiltered was deducted from the total to give values for the bound Ca^{2+} . The volume of fluid removed from the cell in the course of a single experiment was 3% of the total. The between-batch precision of the binding measurements was 1.1% [coefficient of variation (C.V.); $N = 5$]. When Ca^{2+} -bile salt systems were pre-equilibrated at 37°C for 1, 2 or 4 h before filtration, no significant difference in the binding data was found. At the end of each ultrafiltration experiment the contents of the cell were passed through a Millipore 0.22 μm filter and the Ca^{2+} concentration in the filtrate was measured as described above. This was done to check the solubility of Ca^{2+} -bile salt complexes formed during the ultrafiltration experiments.

A calcium electrode (F 2112 Ca) together with a PHM 64 Digital Research pH meter (Radiometer, Copenhagen, Denmark) was used to measure Ca^{2+} activity at 37°C. The between-batch precision of the procedure was 1% (C.V.; $N = 5$). The electrode was only used in solutions containing 3 mM-sodium glycocholate since, at higher bile salt concentrations, the performance of the electrode was affected, making the Ca^{2+} -activity measurement of subsequent standards spuriously low.

Calculation of the dissociation constant (K_D) and the maximum number of binding sites (n) was performed by the method of Wilkinson (1961) by using a programmable calculator (Hewlett-Packard, model 9821A). The calculations depend on the assumption that the binding isotherm takes the form of a rectangular hyperbola; the equation is for a single set of binding sites with no co-operative interactions between sites (Hughes & Klotz, 1956). This assumption was made since it was not possible to define the complete binding curve because of insoluble complex formation at higher Ca^{2+} concentrations.

Preparation and analysis

Sodium glycocholate (Weddell Pharmaceutical Co., London, U.K.) was purified by ethyl acetate extraction (Hofmann, 1963). Final lipid purity was checked by t.l.c. on silica gel G (Anderman and Co.,

London, U.K.) and scanning densitometry (O'Moore & Percy-Robb, 1973) and shown to be 99%. Plates were developed in chloroform/methanol/acetic acid/water (30:10:3:2 by vol.; Kelly & Doisy, 1964) or in 2,2,4-trimethylpentane/di-isopropyl ether/acetic acid (2:1:1, by vol.; Hamilton & Muldrey, 1961), and sprayed with 4-methoxybenzaldehyde (Kritchevsky *et al.*, 1963).

Lecithin (90% egg lecithin; BDH, Poole, Dorset, U.K.) was purified on an alumina column (Singleton *et al.*, 1965). Its final lipid purity was 99% on t.l.c. with a mobile phase of chloroform/methanol/water (65:25:4, by vol.; Marinetti, 1966). I_2 vapour (Sims & Larose, 1962) and Bromothymol Blue (Wagstaff *et al.*, 1974) were used as detection reagents.

Solutions containing sodium glycocholate (120 mM) and phosphatidylcholine (40 mM) were prepared under N_2 and stored for 2 days at 37°C, followed by a further 14 days at 4°C. After this period the glycocholate concentration was 120 mM and the phosphatidylcholine concentration was 30 mM. The decrease in the concentration of phosphatidylcholine was due to the formation of a precipitate that was removed by centrifugation (1500g for 15 min at 22°C). Neither purity nor composition altered after this time. For ultrafiltration experiments the solutions were diluted with Tris/HCl buffer to give a solution containing glycocholate (40 mM) and phosphatidylcholine (10 mM). Ca^{2+} was not detectable in either of the purified lipids.

CaCl_2 and NaCl were AnalaR grade. $^{45}\text{CaCl}_2$ (sp. radioactivity 900 Ci/mol) was from The Radiochemical Centre, Amersham, Bucks., U.K.

Glycocholate concentrations in glycocholate/phosphatidylcholine solutions were measured with 3 α -hydroxy steroid dehydrogenase (Schwartz *et al.*, 1974). Phospholipid concentrations were measured by the method of Bartlett (1959) modified so that hydrolysis was performed with HClO_4 (King, 1932). Ca^{2+} concentrations in the sodium glycocholate solutions were measured with an atomic-absorption spectrophotometer (Instrumentation Laboratory, Lexington, MA, U.S.A.; model 353). Na^+ concentrations were measured by flame photometry with an IL model 343 flame photometer.

Results

Ca^{2+} binding to glycocholate micelles

Estimations of Ca^{2+} binding to glycocholate micelles were made with the ultrafiltration system. The amount of Ca^{2+} bound to glycocholate (Fig. 1) rose as the concentration of the unbound ligand increased. The binding parameters, calculated from the best-fit rectangular hyperbola, were $K_D = 26$ mM and $n = 400$ mmol of Ca^{2+} /mol of glycocholate. After each binding experiment the contents of the

Fig. 1. Ca^{2+} binding to glycocholate (40mM) in Tris/HCl buffer (20mM, pH8.00) containing NaCl (150mM), studied by ultrafiltration

Ca^{2+} concentration in the ultrafiltrate (unbound Ca^{2+}) and in the ultrafiltration cell (total of bound and unbound) was measured and the amount of bound Ca^{2+} calculated by difference.

Fig. 2. Ca^{2+} concentrations in Tris/HCl buffer (20mM, pH8.00) containing NaCl (150mM) and in the same solution containing sodium glycocholate (3mM)

CaCl_2 solution (10mM) in Tris/HCl buffer (20mM, pH8.00) containing NaCl (150mM) was titrated into Tris/HCl buffer and Ca^{2+} activity was measured (ionized calcium in buffer). CaCl_2 solution (10mM) in Tris/HCl buffer (20mM, pH8.00) containing sodium glycocholate (3.0mM) and NaCl (150mM) was titrated into Tris/HCl buffer containing sodium glycocholate (3mM) and Ca^{2+} activity was measured (ionized calcium in glycocholate).

cell were passed through the $0.22\mu\text{m}$ filters. The mean recovery of Ca^{2+} was 98.3% (range 97–101%, $N=10$), confirming the solubility of the calcium-glycocholate micelle complexes formed.

Fig. 3. Reversibility of Ca^{2+} binding to glycocholate (40mM) in Tris/HCl buffer (20mM, pH8.00) containing NaCl (150mM)

(a) Ca^{2+} binding to glycocholate (40mM) in Tris/HCl buffer (20mM, pH8.00) containing NaCl (150mM) studied in a constant-volume ultrafiltration cell with the reservoir containing CaCl_2 (4.59mM) and sodium glycocholate (4.0mM). Ca^{2+} concentration in the ultrafiltrate and the volume of the ultrafiltrate were measured and Ca^{2+} binding was calculated (Williamson *et al.*, 1978). (b) The contents of the reservoir were replaced with Tris/HCl buffer (20mM, pH8.00) containing NaCl (150mM) and sodium glycocholate (4.0mM) and further fractions of ultrafiltrate were collected for measurement of Ca^{2+} concentration; the Ca^{2+} remaining bound to glycocholate micelles was calculated.

Behaviour of Ca^{2+} in submicellar solutions of sodium glycocholate

Sodium glycocholate (3mM) in Tris/HCl buffer was used to produce a solution saturated with unassociated bile salt, but containing few micelles: the critical micellar concentration of sodium glyco-

Fig. 4. Effects of pH, NaCl concentration and phosphatidylcholine on Ca^{2+} binding to glycocholate (40 mM) in Tris/HCl buffer (20 mM, pH 8.00) studied by ultrafiltration

In (a) the NaCl concentration was 150 mM and the effect of changing pH was studied by using Tris/HCl buffer at pH 8.00 ($\circ$) and 7.20 ($\bullet$). In (b) the pH was 8.00 and various concentrations (0–250 mM) of NaCl were used. In (c) the pH was 8.00, the NaCl concentration was 150 mM; Ca^{2+} binding was studied in the absence ($\circ$) and presence of phosphatidylcholine (10 mM) ($\bullet$). The concentration of Ca^{2+} in the ultrafiltrate (unbound) and in the ultrafiltration cell (total of bound and unbound) was measured and the amount of bound Ca^{2+} was obtained by difference.

cholate in the presence of NaCl (150 mM) is in the range 3–6 mM (Small, 1971). CaCl_2 was titrated into this sodium glycocholate-containing solution and into Tris/HCl buffer alone. Ca^{2+} activity in these solutions measured with the calcium electrode was not changed by the presence of the bile salt (Fig. 2).

On the basis of these two series of experiments it was concluded that, in aqueous solutions, (1) the calcium ligand (concentration range 0–5 mM) did not associate with sodium glycocholate in the form of its monomer, (2) the association of the ligand was with glycocholate micelles, and (3) the Ca^{2+} –glycocholate complex formed was soluble. On the assumption of an average glycocholate-aggregation number of 5 molecules (Small, 1971), a maximum of 2 Ca^{2+} ions was bound to each micelle.

Reversibility of Ca^{2+} binding to glycocholate micelles

With the ultrafiltration cell in the constant-volume mode, binding of Ca^{2+} to glycocholate micelles was confirmed (Fig. 3a). This bound Ca^{2+} was washed out of the cell progressively when the solution in the reservoir was replaced by Tris/HCl buffer containing sodium glycocholate, but no CaCl_2 (Fig. 3b). During the addition and removal of Ca^{2+} , the amount of Ca^{2+} bound was similar at corresponding concentrations of free ligand. The interaction between Ca^{2+} and glycocholate micelles was therefore rapidly and stoichiometrically reversible.

Effects of pH on binding of Ca^{2+} to glycocholate micelles

The mass of Ca^{2+} bound to glycocholate micelles was lower at pH 7.20 than at pH 8.00 (Fig. 4a). Throughout the range of concentrations used, Ca^{2+} binding was decreased by approx. 40%. The binding parameters at pH 7.20, calculated from the best-fit hyperbola, were $K_D = 95$ mM and $n = 150$ mmol of Ca^{2+} /mol of glycocholate.

Effect of counter-ion concentration on binding of Ca^{2+} to glycocholate micelles

Ca^{2+} binding to glycocholate micelles increased as the NaCl concentration decreased (Fig. 4b). This effect was most marked at NaCl concentrations below 150 mM; 70% more Ca^{2+} was bound in the presence of 50 mM NaCl than in the presence of 150 mM NaCl.

Effect of phosphatidylcholine on Ca^{2+} binding to glycocholate micelles

The addition of phosphatidylcholine (10 mM) increased Ca^{2+} binding to glycocholate micelles (40 mM) (Fig. 4c). Binding was increased by 66% and by 43% at free Ca^{2+} concentrations of 1.0 and 2.0 mM respectively.

Discussion

The present studies have demonstrated an association between Ca^{2+} ions and glycocholate micelles that is rapidly reversible. It is affected by the incorporation of phosphatidylcholine into the micelles as well as by the pH and ionic strength of the system.

Bile salt micelles are not saturated with Ca^{2+} at the concentrations found in human bile (1–12mM in gallbladder bile; Nakayama & Van der Linden, 1970). However, to calculate the binding parameters K_D and n , we have made the assumption that the binding isotherm takes the form of a rectangular hyperbola, which is the equation for a single set of non co-operative binding sites (Hughes & Klotz, 1956). We made this assumption because of the known homogeneity of micellar structure at the bile salt concentrations used in the present paper (Small, 1968), because we have shown that unaggregated bile salt does not bind significant quantities of Ca^{2+} , and also because the data fit well into a rectangular hyperbola.

Introduction of the amphiphile phosphatidylcholine into glycocholate micelles produces an increase in the binding of Ca^{2+} to the micelles (Fig. 4c). By contrast, when the amphiphile hexanol is introduced into micelles of sodium dodecyl sulphate, Ca^{2+} ions are released (Pearson & Lawrence, 1967). Ekwall *et al.* (1952) and Biswas & Mukerji (1960) have also demonstrated an increase in conductance in detergent solutions after the introduction of alcohols, and have interpreted this as release of counter ions. Phosphatidylcholine, although comparable with linear aliphatic alcohols as a polar amphiphile, possesses two highly polar groups of opposite charge, namely phosphate and quaternary nitrogen. The phosphate group has been shown to bind Ca^{2+} ions strongly in the structurally related molecule, phosphatidylserine (Abramson *et al.*, 1964). By analogy with this, the effect of phosphatidylcholine on Ca^{2+} binding to glycocholate micelles may be due to Ca^{2+} binding to the phosphate group of phosphatidylcholine.

NaCl decreases the amount of Ca^{2+} bound to the glycocholate micelles (Fig. 4b). Binding of Na^+ and K^+ ions to bile salt micelles has been demonstrated (Moore & Dietschy, 1964), and a fall in NaCl concentration is accompanied by a decrease in the size of the micelles with a consequent increase in their numbers (Carey & Small, 1969). The effect of NaCl on Ca^{2+} binding may therefore be due to competition between Na^+ and Ca^{2+} ions for negatively charged binding sites on the surface of the micelles, and to effects on micellar surface area. Ca^{2+} binding was also decreased by a change of pH from 8.00 to 7.20 (Fig. 4a). This is unlikely to be due to a direct competitive effect since the concentration of Ca^{2+} in bile is approximately 100 000 times greater than the H^+ ion concentration.

We have shown that the dissociation constant for the interaction between Ca^{2+} and glycocholate is 24mM at pH 8.00 whereas the dissociation constants of the highly insoluble salts, CaCO_3 and calcium phosphate are 1.0mM and 2.0mM respectively (Greenwald, 1941; Walser, 1961). It would seem therefore, that the glycocholate- Ca^{2+} interactions are of sufficient affinity to compete with less soluble complexes for Ca^{2+} in bile. Bile obtained from patients under fasting conditions is supersaturated with respect to cholesterol (Northfield & Hofmann, 1975). This supersaturation is associated with low bile salt-secretion rates. It is not known whether Ca^{2+} secretion also changes under fasting conditions, thus affecting the degree of interaction between Ca^{2+} and bile salt micelles and therefore the potential for formation of insoluble Ca^{2+} salts.

Binding of Na^+ and K^+ ions to glycocholate micelles is probably not important in the formation of gallstones, since salts containing these ions are soluble. Ca^{2+} salts are less soluble and, in contrast with Na^+ and K^+ salts, are important constituents of gallstones (Sutor & Wooley, 1973). With the exception of the Ca^{2+} salt of lithocholic acid, traces of which have been reported (Schoenfield *et al.*, 1966), bile salts are rarely found in human gallstones.

In the present studies the complexes of Ca^{2+} with glycocholate that did form were shown to be soluble when the Ca^{2+} concentrations were similar to those found in bile. This may explain why bile salts are absent from gallstones. Since complexes of glycocholate with Ca^{2+} are soluble, and since their formation decreases the activity of the Ca^{2+} in solution, binding of Ca^{2+} may act as a mechanism that decreases a tendency for the formation in bile of insoluble Ca^{2+} salts and their subsequent growth in crystalline form.

During these studies B. W. A. W. was supported by a Research Fellowship from the University of Edinburgh Faculty of Medicine.

References

- Abramson, M. B., Katzman, R. & Gregor, H. P. (1964) *J. Biol. Chem.* **239**, 70–76
- Admirand, W. H. & Small, D. M. (1968) *J. Clin. Invest.* **47**, 1043–1052
- Bartlett, G. R. (1959) *J. Biol. Chem.* **234**, 466–468
- Biswas, A. K. & Mukherji, B. K. (1960) *J. Phys. Chem.* **64**, 1–4
- Carey, M. C. & Small, D. M. (1969) *J. Colloid Sci.* **31**, 382–396
- Ekwall, P., Danielsson, I. & Henrikson, S. (1952) *Acta Chem. Scand.* **6**, 1297–1298
- Greenwald, I. (1941) *J. Biol. Chem.* **141**, 789–796
- Hamilton, J. G. & Muldrey, J. E. (1961) *J. Am. Oil Chem. Soc.* **38**, 582–585
- Hofmann, A. F. (1963) *Biochem. J.* **89**, 57–58
- Hofmann, A. F. & Small, D. M. (1967) *Annu. Rev. Med.* **18**, 333–376

- Hughes, T. R. & Klotz, I. M. (1956) *Methods Biochem. Anal.* **3**, 265–299
- Kelly, R. L. & Doisy, E. A. (1964) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **23**, 173
- King, E. J. (1932) *Biochem. J.* **26**, 292–297
- Kritchevsky, D., Martak, D. S. & Rothblat, G. H. (1963) *Anal. Biochem.* **5**, 388–392
- Marinetti, G. V. (1966) *J. Lipid Res.* **3**, 1–20
- Moore, E. W. & Dietschy, J. M. (1964) *Am. J. Physiol.* **206**, 1111–1117
- Nakayama, F. & Van der Linden, W. (1970) *Acta Chem. Scand.* **136**, 605–610
- Northfield, T. C. & Hofmann, A. F. (1975) *Gut* **16**, 1–17
- O'Moore, R. R. L. & Percy-Robb, I. W. (1973) *Clin. Chim. Acta* **43**, 39–47
- Pearson, J. L. & Lawrence, A. S. C. (1967) *Trans. Faraday Soc.* **63**, 488–494
- Schoenfield, L. J., Sjövall, J. & Sjövall, K. (1966) *J. Lab. Clin. Med.* **68**, 186–194
- Schwartz, H. P., Bergmann, K. V. & Paumgartner, G. (1974) *Clin. Chim. Acta* **50**, 197–206
- Sims, R. P. A. & Larose, J. A. G. (1962) *J. Am. Oil Chem. Soc.* **39**, 232
- Singleton, W. S., Gray, M. S., Brown, M. L. & White, J. L. (1965) *J. Am. Oil Chem. Soc.* **42**, 53–56
- Small, D. M. (1968) *Adv. Chem. Ser.* **84**, 31–52
- Small, D. M. (1971) in *The Bile Acids* Nair, P. P. & Kritchevsky, D., eds.), vol. 1, pp. 249–356, Plenum Press, London
- Strange, R. C., Nimmo, I. A. & Percy-Robb, I. W. (1976) *Biochem. J.* **156**, 427–433
- Sutor, D. J. & Wooley, S. E. (1973) *Gut* **14**, 215–220
- Wagstaff, T. I., Whyley, G. A. & Freedman, G. (1974) *Ann. Clin. Biochem.* **11**, 24–30
- Walser, M. (1961) *J. Clin. Invest.* **40**, 723–730
- Watkinson, G. (1967) *Proc. World Congr. Gastroenterol.* **3rd**, vol. 4, pp. 157–162, Karger, Basel
- Wilkinson, G. N. (1961) *Biochem. J.* **80**, 324–332
- Williamson, B. W. A., Strange, R. C. & Percy-Robb, I. W. (1978) *Biophys. Acta* **543**, 397–402