# BINDING OF CALCIUM BY CELLULOSE MEMBRANES AND SEPHADEX

K.C. Reed

Department of Biochemistry, School of General Studies,
Australian National University,
Canberra, A.C.T., Australia.

Received January 15,1973

Summary: Calcium ions bind to polyglucose matrices (dialysis membranes and Sephadex) with low affinity, but in amounts sufficient to markedly influence the rate of dialysis of $Ca^{++}$. In applications where such interaction is critical (binding studies by flow dialysis or ultrafiltration), $Ca^{++}$ binding can be largely prevented by acetylation of the membrane and/or high salt concentration.

## INTRODUCTION

Numerous reports have appeared recently on the isolation and characterisation of calcium binding fractions and proteins derived from muscle (1-4), intestinal mucosa (5,6), kidney (7), cartilage and connective tissue (9), retinal discs (10), brain (11,12), serum (13) and mitochondria (14-16). The binding properties of these various fractions have in some cases been determined by dialysis techniques, either by conventional equilibrium dialysis or the more rapid and, in some ways, more accurate flow dialysis technique of Colowick and Womack (17). It is perhaps timely to point out that the use of unmodified cellulose membranes in such studies can result in quite serious errors due to binding by the membrane itself. Additional experiments have indicated that all polyglucose matrices bind calcium.

## MATERIALS AND METHODS

The flow dialysis apparatus used in these experiments had a lower chamber volume of 0.25 ml and a maximum

volume of 15 ml in the upper chamber. The membrane area was 0.64 $cm^2$. Fractions of 290 µl were collected and 250 µl aliquots counted by liquid scintillation. The cellulose membranes were obtained from Thomas Scientific Instrument Company (#4465-A2), although similar results were obtained with all grades of dialysis tubing tested. The binding of $^{45}Ca^{++}$ to membranes was measured directly by incubating 1 $cm^2$ pieces of membrane in beakers with $^{45}Ca^{++}$ and stirring gently. After a specified time interval, the membranes were removed, rinsed three times in distilled water, blotted dry and counted by liquid scintillation.

## RESULTS AND DISCUSSION

The artefacts resulting from $Ca^{++}$ adsorption to cellulose are illustrated by the results of the "control" flow dialysis experiment shown in Figure 1A. Theory predicts (17) that the concentration of $^{45}Ca^{++}$ appearing in the dialysate should plateau within 5 x (volume of the lower chamber). In practice, the plateau is reached much later at about 15 x (volume of lower chamber). The addition of a large excess of $^{40}Ca^{++}$ results in a sharp peak of radioactivity in the dialysate, followed by a decline to a level much lower than the initial value; a second addition of $^{40}Ca^{++}$ causes a further decrease in the plateau value. Since the rate of dialysis decreases with increasing $Ca^{++}$ concentration, this system is totally unsatisfactory for binding analyses.

The phenomena apparent in Fig. 1A are explicable in terms of the binding properties of the membrane. Firstly, direct measurements showed the binding to be time de-

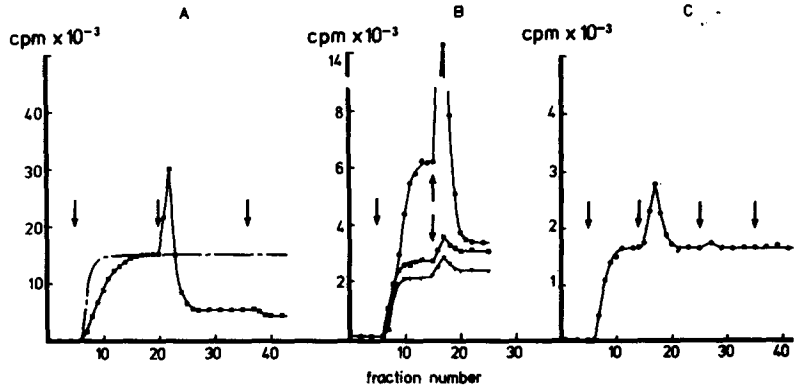


Fig. 1. Flow dialysis profiles with $^{45}Ca^{++}$. All experiments were done at 4°. The flow buffer was 5mM tris-chloride, pH 7.4, and the fraction volume 0.29 ml. The ordinate is cpm/250 µl aliquot of the appropriate fraction.
A. Upper chamber: 15 ml of 5mM tris-chloride, pH 7.4. Flow rate 0.204 ml/min. At the first arrow 5 nmoles $^{45}Ca^{++}$ (10 µCi) was added; subsequent arrows show the addition of 10 µmoles $CaCl_2$. The dashed line represents the theoretical profile.
B. Upper chamber:
● 5 ml of 0.25M sucrose, 10mM Hepes, pH 7.4
■ 5 ml of 0.1M NaCl, 10mM Hepes, pH 7.4
▼ 5 ml of 0.1 M KCl, 10 mM Hepes, pH 7.4

In each case the flow rate was 0.673 ml/min. At the first arrow 2.5 nmoles $^{45}Ca^{++}$ (5 µCi) was added; the second arrow shows the addition of 100 µmoles $CaCl_2$.
C. Upper chamber: 5 ml of 5mM tris-chloride, pH 7.4. Acetylated membrane. Flow rate 0.619 ml/min. At the first arrow 2.5 nmoles $^{45}Ca^{++}$ (5 µCi) was added; subsequent arrows show the addition of 100 µmoles $CaCl_2$.

---

pendent, requiring about 20 minutes for maximal adsorption of $^{45}Ca^{++}$. Similarly, a large proportion of the $^{45}Ca^{++}$ bound at this time exchanged but slowly with $^{40}Ca^{++}$. These time-dependent properties probably reflect the time taken for $Ca^{++}$ to diffuse to internal sites in the membrane. Secondly, the binding was characteristic of the presence of a very large number of low affinity sites, in that approximately 1% of the added $^{45}Ca^{++}$ was bound/cm$^2$

membrane over a wide range of calcium concentrations. This is further supported by the observation that 100 mM NaCl or KCl inhibited the binding by more than 90%. The slow attainment of plateau in the flow dialysis profile thus represents the time-dependent equilibration of $^{45}Ca^{++}$ with membrane binding sites. Addition of excess $^{40}Ca^{++}$ displaces the previously bound $^{45}Ca^{++}$ and virtually saturates all available sites. The resultant net positive charge associated with the membrane causes a decrease in the dialysis rate due to charge repulsion (cf. Fig. 1B; dialysis rates in the presence of NaCl and KCl are similar to that with excess $Ca^{++}$).

Pre-treatment of the membrane by boiling in 0.1M $KHCO_3$ resulted in only a slight decrease in the amount of $^{45}Ca^{++}$ bound. Most of the binding is thus an intrinsic property of the cellulose material, and is almost certainly due to the hydroxyl moieties of the glucose residues.

In terms of the use of these membranes in flow dialysis experiments, most of the difficulties are sufficiently overcome by using NaCl or KCl media (Fig. 1B). However, these media have the obvious disadvantage of similarly inhibiting low affinity binding of $Ca^{++}$ to the proteins whose properties are being studied. Specific high affinity binding may also be at least partially inhibited.

To overcome these limitations, small pieces of membrane were extensively acetylated by treatment at 70° for 17½ hours in 25% acetic anhydride/pyridine. The modified membranes were rinsed thoroughly in 0.1N acetic acid

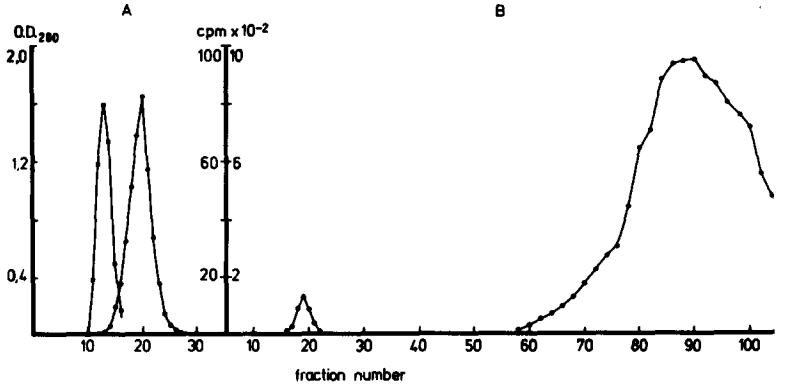


Fig. 2. Sephadex elution profiles.
A. 6.0 ml aqueous extract of rat liver mitochondria (19) containing 2mM EDTA and 5 µCi $^{45}Ca^{++}$ was applied to a column of coarse Sephadex G-25 (1.6 x 36 cm) and eluted with 5mM tris-chloride (pH 7.4) at 4°.

● cpm/10 µl aliquot of appropriate fraction
■ absorbance at 280 nm (1 cm lightpath).

B. 4.0 ml aqueous extract of rat liver mitochondria containing 5 µCi $^{45}Ca^{++}$ was applied to a column of coarse Sephadex G-25 (1.6 x 47 cm) and eluted with 20mM tris-chloride (pH 7.4) at 4°. Fraction volume was 1.73 ml and the flow rate 0.99 ml/min. The first peak of radioactivity is due to $Ca^{++}$ associated with protein.

● cpm/10 µl aliquot of appropriate fraction.

---

and water and stored in 50% ethanol at 0°. Before use, they were rinsed in water and stretched in both directions. The binding of $^{45}Ca^{++}$ by membranes treated in this way is very low and rapidly exchangeable. Figure 1 shows a control flow dialysis experiment using such a membrane. The peak following the first addition of 20 mM $^{40}Ca^{++}$ indicates the small amount of $^{45}Ca^{++}$ bound to the membrane (approx. 0.02% of total compared with 0.4% of total for unmodified membranes, Fig. 1A), but this is the only artefact apparent. Most significantly, there is no change in the rate of dialysis. Thus, acetylated cellu-

lose membranes provide a far superior semi-permeable barrier for $Ca^{++}$ binding applications. They are virtually essential in studies employing rates of dialysis (cf. 8,16) or ultrafiltration (18), although conventional equilibrium dialysis studies would also be less ambiguous with these membranes.

The binding of $Ca^{++}$ to glucose residues was also encountered in experiments designed to remove endogenous $Ca^{++}$ from aqueous extracts of rat liver mitochondria. A trace of $^{45}Ca^{++}$ was added to a sample of extract and the mixture applied to a column of Sephadex G-25. When the extract also contained EDTA, all of the $^{45}Ca^{++}$ appeared in a sharp peak corresponding to the bed volume (Fig. 2A). In the absence of EDTA, most of the label appeared as a very broad peak centred at about three times the bed volume (Fig. 2B). Inclusion of 0.1M glucose in the elution buffer caused a more rapid displacement of $^{45}Ca^{++}$ from the column.

It thus appears that the binding of $Ca^{++}$ to carbohydrate moieties is a general phenomenon which should be taken into account in all applications where such interaction is likely to occur.

## REFERENCES

1. MacLennan, D.H. and Wong, P.T.S., Proc. Natl. Acad. Sci., 68, 1231 (1971).
2. Bertrand, H.A., Masoro, E.J., Ohnishi, T. and Yu, B.P., Biochem., 10, 3679 (1971)
3. Drabikowski, W. and Barytko, B., Acta Biochim. Polon., 18, 353 (1971).
4. Nockolds, C.E., Kretsinger, R.H., Coffee, C.J. and

Bradshaw, R.A., Proc. Natl. Acad. Sci., 69, 581 (1972).

5. Ingersoll, R.J. and Wasserman, R.H., J. Biol. Chem., 246, 2808 (1971).
6. Drescher, D. and DeLuca, H.F., Biochem., 10, 2302 (1971).
7. Piazolo, P., Schleyer, M. and Franz, H.E., Z. Physiol. Chem., 352, 1480 (1971).
8. Vittur, F., Pugliarello, M.C., and de Bernard, B., Biochem. Biophys. Res. Comm., 48, 143 (1972).
9. Urry, D.W., Proc. Natl. Acad. Sci., 68, 810 (1971).
10. Neufeld, A.H., Miller, W.H. and Bitensky, M.W., Biochim. Biophys. Acta, 266, 67 (1972).
11. Wolff, D.J. and Siegel, F.L., Arch. Biochem. Biophys., 150, 578 (1972).
12. Wolff, D.J. and Siegel, F.L., J. Biol. Chem., 247, 4180 (1972).
13. Ansari, A.Q., Dolphin, P.J., Lazier, C.B., Munday, K.A. and Akhtar, M., Biochem. J., 122, 107 (1971).
14. Lehninger, A.L., Biochem. Biophys. Res. Comm., 42, 312 (1971).
15. Gomez-Puyou, A., de Gomez-Puyou, M.T., Becker, G. and Lehninger, A.L., Biochem. Biophys. Res. Comm., 47, 814 (1972).
16. Sottocasa, G., Sandri, G., Panfili, E., de Bernard, B., Gazzotti, P., Vasington, F.D. and Carafoli, E., Biochem. Biophys. Res. Comm., 47, 808 (1972).
17. Colowick, S.P. and Womack, F.C., J. Biol. Chem., 244, 774 (1969).
18. Paulus, H., Analyt. Biochem., 32, 91 (1969).
19. Caplan, A.I. and Greenawalt, J.W., J. Cell Biol., 31, 455 (1966).