SECTION-V

CHELEX PARTITION METHOD: DERIVATION OF EQUATIONS

This is based on the Ph.D. Thesis of R. E. Godt (1971):

Calcium-activated tension of skinned muscle fibres: dependence on Mg-ATP concentration. Ph.D. Thesis, University of Washington, Seattle, 128 pp.

CONTENTS

PARTITION METHOD

1) EGTA AND CALCIUM

Introduction: In a Ca^{2+} buffer solution with only one Ca^{2+} binding ligand present, the following holds:

$$[Ca]_T = [Ca^{2+}] + [Ca-EGTA^{2-}] \quad [1]$$

The problem is to measure $[Ca^{2+}]$ and $[Ca\text{-}EGTA^{2-}]$.

Resin method: If an insoluble Ca^{2+}-binding resin is added to the mixture and the solution shaken vigorously for sufficient time, Ca^{2+} will be bound not only by EGTA, but also by the resin:

$$[Ca]_T = [Ca^{2+}] + [Ca-EGTA^{2-}] + [Ca-R] \quad [2]$$

However, the resin is insoluble and can be precipitated by centrifugation and the [Ca-R] measured. The usual way to do this is to add Ca^{45} to the solution and the Ca^{45} is measured in both supernatant and precipitate. From these measurements [Ca-R] can be estimated:

$$[Ca-R] = [Ca]_T * \frac{Ca^{45}(\text{precipitate})}{\{Ca^{45}(\text{precipitate}) + Ca^{45}(\text{supernatant})\}} \quad [3]$$

or

$$[Ca-R] = [Ca]_T * \frac{100\%Ca^{45} - \%Ca^{45}(\text{supernatant})}{100\%Ca^{45}} \quad [4]$$

The function $\frac{100\%Ca^{45} - \%Ca^{45}(\text{supernatant})}{100\%Ca^{45}}$ is simply the fraction of total Ca^{45} in the precipitate.

Knowing the [Ca-R] allows an estimation of the $[Ca^{2+}]$ because, if the [Ca-R] << $[R]_T$ the relationship between the two can be regarded as linear and:

$$[Ca^{2+}] = s_R * [Ca\text{-}R] \quad [5]$$

where s_R is the slope of the linear relationship.

The relationship is not linear, but hyperbolic, the actual binding curve being:

$$[Ca-R] = \frac{[R]_T[Ca^{2+}]}{([Ca^{2+}] + K_R)} \text{ and } K_R = \frac{[Ca^{2+}][R^{2-}]}{[Ca-R]} \quad [6][7]$$

Rearranging equation [6] to give $[Ca^{2+}]$ in terms of [Ca-R]:

$$[Ca^{2+}] = [Ca-R]\frac{K_R}{([R]_T - [Ca-R])} \quad [8]$$

When $[Ca\text{-}R] << [R]_T$ equation [8] becomes:

$$[Ca^{2+}] = [Ca-R]\frac{K_R}{[R]_T} \text{ or } [Ca^{2+}] = [Ca\text{-}R] \ ^*s_R \quad [9][10]$$

where:

$$s_R = \frac{K_R}{[R]_T} \quad [11]$$

Linear Assumption

To test the linear assumption, calculations were carried out on a theoretical resin which had a K_R of 0.0003 mmol/l (similar to EGTA) and a concentration of 4 mmol/l. $[Ca^{2+}]$ was calculated using either equation [8] (from [Ca-R]) or from equation [9] (linear assumption). The results are illustrated in Figure 1.

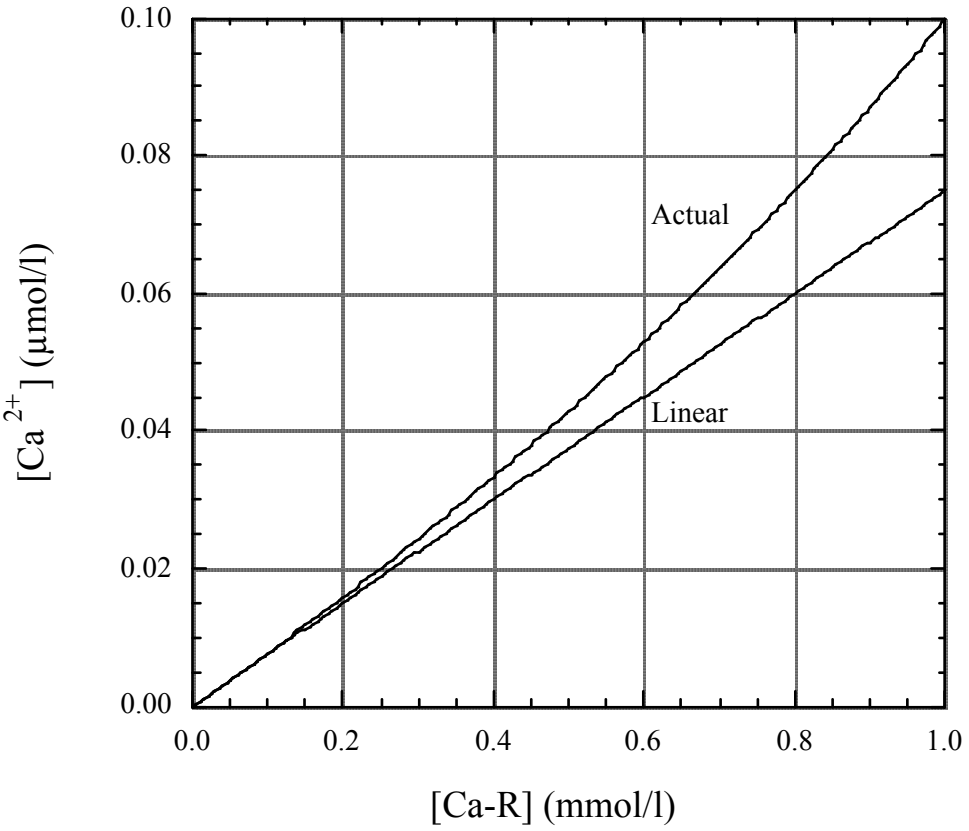

Figure 1: Graph to show the difference between assuming a linear relationship between $[Ca^{2+}]$ and [Ca-R] and the actual hyperbolic relationship.

As illustrated in Figure 1, the linear assumption is a good approximation up to a [Ca-R] of 0.2 mmol/l, thereafter it diverges. This value of [Ca-R] corresponds to a $[Ca]_T$ of 200 µmol/l.

Estimation of slope s_R

This can be determined in separate experiments with no EGTA present only resin. Under these circumstances:

$$s_R = \frac{Ca^{45}(\text{supernatant})}{Ca^{45}(\text{precipate})} \text{ or } s_R = \frac{\%Ca^{45}(\text{sup erna tan t})}{100\% - \%Ca^{45}(\text{sup erna tan t})} \qquad [12][13]$$

Actual experiment:

1) The constant s_R is determined in the background solution, but only with the resin present, equation [13].
2) With both EGTA and resin present the [Ca-R] is determined at various $[Ca]_T$ using equation [4].
3) If [Ca-R] is known then $[Ca^{2+}]$ can be calculated using equation [5]
4) It then follows from equation [2] that:

$$[Ca-EGTA^{2-}] = [Ca]_T - ([Ca^{2+}] + [Ca-R]) \qquad [14]$$

5) Evaluation of the results can be carried out using some linearisation of the hyperbolic binding curve, e.g. a Scatchard plot.

2) EGTA AND A SECOND CALCIUM BINDING LIGAND

Resin method: The complication in this method is that the binding of Ca^{2+} to EGTA is to be measured in the presence of another Ca^{2+} binding ligand L. Under these circumstances:

$$[Ca]_T(EGTA) = [Ca^{2+}] + [Ca-EGTA] + [Ca-L] \qquad [15]$$

or

$$[Ca]_T(EGTA) = [Ca-EGTA] + ([Ca-L] + [Ca^{2+}]) \qquad [16]$$

The trick is to estimate ($[Ca^{2+}]$ + [Ca-L] + [Ca-R]) with the resin method with no EGTA in the solution. The assumption for this method is that, like the resin the relationship between [Ca-L] and $[Ca^{2+}]$ is also linear. If this is so then:

$$[Ca^{2+}] = s_L * [Ca\text{-}L] \qquad [17]$$

With the resin method:

$$[Ca]_T(\text{No EGTA}) = [Ca^{2+}] + [Ca-L] + [Ca-R] \qquad [18]$$

substituting equations [5] and [17] in equation [18] gives:

$$[Ca]_T(\text{No EGTA}) = [Ca^{2+}] + \frac{[Ca^{2+}]}{s_L} + \frac{[Ca^{2+}]}{s_R} \quad [19]$$

or

$$[Ca]_T(\text{No EGTA}) = [Ca^{2+}](1 + \frac{1}{s_L} + \frac{1}{s_R}) \quad [20]$$

At a given [Ca-R] and [Ca-L] there is a linear relationship between $[Ca]_T(\text{No EGTA})$ and [Ca^{2+}]. At a constant $[L]_T$ it is also possible to vary the $[R]_T$. Substituting for [Ca^{2+}] from equation [5] in equation [20] gives:

$$[Ca]_T(\text{No EGTA}) = [Ca-R]*s_R*(1 + \frac{1}{s_L} + \frac{1}{s_R}) \quad [21]$$

or

$$\frac{[Ca]_T(\text{No EGTA})}{[Ca-R]} = s_R*(1 + \frac{1}{s_L} + \frac{1}{s_R}) \quad [22]$$

and

$$[Ca]_T(\text{No EGTA}) = s_T(\text{No EGTA})*[Ca-R] \quad [23]$$

where

$$s_T(\text{No EGTA}) = s_R*(1 + \frac{1}{s_L} + \frac{1}{s_R}) \quad [24]$$

and

$$s_T(\text{No EGTA}) = \frac{[Ca]_T(\text{No EGTA})}{[Ca-R]} \quad [25]$$

Equation [23] means that a given [Ca^{2+}], since [Ca-L] equals s_L*[Ca^{2+}] and [Ca-R] equals s_R*[Ca^{2+}], the sum of ([Ca^{2+}] + [Ca-L] + [Ca-R]) is a linear function of the [Ca-R]. In other words, even if EGTA is added to the solution, at a given [Ca^{2+}], the sum of ([Ca^{2+}] + [Ca-L] + Ca-R) can be estimated from equation [23] provided s_T(No EGTA) is known.

In the resin method s_T(No EGTA) can be estimated if the resin is added to the background solution with the ligand but with no EGTA. Under these circumstances:

$$s_T(\text{No EGTA}) = \frac{\{Ca^{45}(\text{precipate}) + Ca^{45}(\text{supernatant})\}}{Ca^{45}(\text{precipate})} \quad [26]$$

or

$$s_T(\text{No EGTA}) = \frac{100\%Ca^{45}}{100\%Ca^{45} - \%Ca^{45}(\text{supernatant})} \quad [27]$$

Actual experiment:

1) The constant s_R is determined in the background solution, but only with the resin present, equation [13].
2) The constant s_T(No EGTA) is determined in the background solution with resin and the ligand L present, equation [27].
3) In the background solution, with ligand, EGTA and resin present, the [Ca-R] is determined at various $[Ca]_T$ using equation [4].
4) Since [Ca-R] is known then $[Ca^{2+}]$ can be calculated using equation [5]
5) From the [Ca-R] the sum of ($[Ca^{2+}]$ + [Ca-L] + [Ca-R]) namely, $[Ca]_T$(No EGTA) is calculated from equation [23].
6) The [Ca-EGTA] can now be calculated as follows. With ligand, EGTA and resin present in the background solution:

$$[Ca]_T(EGTA) = [Ca{-}EGTA] + ([Ca^{2+}] + [Ca{-}L] + [Ca{-}R]) \qquad [28]$$

It follows that:

$$[Ca{-}EGTA] = [Ca]_T(EGTA) - ([Ca^{2+}] + [Ca{-}L] + [Ca{-}R]) \qquad [29]$$

and it follows that:

$$[Ca{-}EGTA] = [Ca]_T(EGTA) - [Ca]_T(No\ EGTA) \qquad [30]$$

7) Evaluation of the results can be carried out using some linearisation of the hyperbolic binding curve, e.g. a Scatchard plot.

DERIVATION OF EQUATIONS

EQUATIONS

For simplicity the equations have been developed for calcium binding to only one ligand, namely EGTA. The case in which two calcium binding ligands, (EGTA and another) are present in the solution is not considered.

1) [Ca^{2+}] in a solution of calcium, and ligand *or* resin:

The quadratic equations for this have been developed in Section-I

2) [Ca^{2+}] in a solution of calcium, resin *and* ligand

In such a solution:

$$[Ca]_T = [Ca^{2+}] + [Ca-R] + [Ca-L] \qquad [28]$$

$$[R]_T = [Ca-R] + [R^{2-}] \qquad [29]$$

$$[L]_T = [Ca-L] + [L^{2-}] \qquad [29]$$

At any given [Ca^{2+}] the bound concentration is given by equation [141], page 23, Section-I:

$$[Ca-R] = \frac{[R]_T[Ca^{2+}]}{([Ca^{2+}] + K_R)} \text{ and } [Ca-L] = \frac{[L]_T[Ca^{2+}]}{([Ca^{2+}] + K_L)} \qquad [30][31]$$

substituting equations [30] and [31] in equation [28] gives:

$$[Ca]_T = [Ca^{2+}] + \frac{[R]_T[Ca^{2+}]}{([Ca^{2+}] + K_R)} + \frac{[L]_T[Ca^{2+}]}{([Ca^{2+}] + K_L)} \qquad [32]$$

This equation has now to be solved for [Ca^{2+}]. Simplification gives a cubic of the form:

$$A[Ca^{2+}]^3 + B[Ca^{2+}]^2 + C[Ca^{2+}] + D = 0 \qquad [33]$$

where

$$A = 1$$

$$B = (K_R + K_L + [R]_T + [L]_T - [Ca]_T)$$

$$C = (K_R K_L + [R]_T K_L + [L]_T K_R - K_R[Ca]_T - K_L[Ca]_T \qquad [34]$$

$$D = -[Ca]_T K_R K_L$$

THEORETICAL EXPERIMENT

INITIAL CONDITIONS

How accurate is the method and what are its disadvantages? In order to try to answer these questions the pK_{app} and purity was calculated in a theoretical experiment. The initial conditions are somewhat arbitrary, but were chosen for the purpose of illustration. They were:

Ligand

$[EGTA]_T$ = 1 mmol/l

pK_{app} = 6.537

Resin

$[Resin]_T$ = 1 mmol/l (purely arbitrary)

K_R = 0.125 mmol/l

In Godt's Thesis (1971) $\frac{[Ca^{2+}]}{[Ca-R]}$ is in round figures equal to 0.125.

From equation [9]

$$[Ca^{2+}] = \frac{K_R}{[R]_T}[Ca-R]$$

Since $[R]_T$ was chosen to be 1 mmol/l, this makes K_R 0.125.

Maximal $[Ca]_T$

This were 5 µmol/l, 10 µmol/l, and then in steps of 10 µmol/l to 100 µmol/l. For each maximal $[Ca]_T$, 10 equal steps in concentration were chosen.

CALCULATIONS

These were carried out as follows:

Step 1: Estimate s_R

1) For each maximal $[Ca]_T$, $[Ca^{2+}]$ was estimated from the following quadratic equation:

$$[Ca^{2+}] = \frac{-(K_R + [R]_T - [Ca]_T) + \sqrt{(K_R + [R]_T - [Ca]_T)^2 + 4 * K_R[Ca]_T}}{2}$$

2) [Ca-R] was calculated as the difference ($[Ca]_T$ - Ca^{2+}])

3) [Ca-R] (x-axis) was plotted against $[Ca^{2+}]$ (y-axis) and a linear regression line was drawn through the points. The procedure is illustrated in Figure 2 for a $[Ca]_T$ of 100 µmol/l.

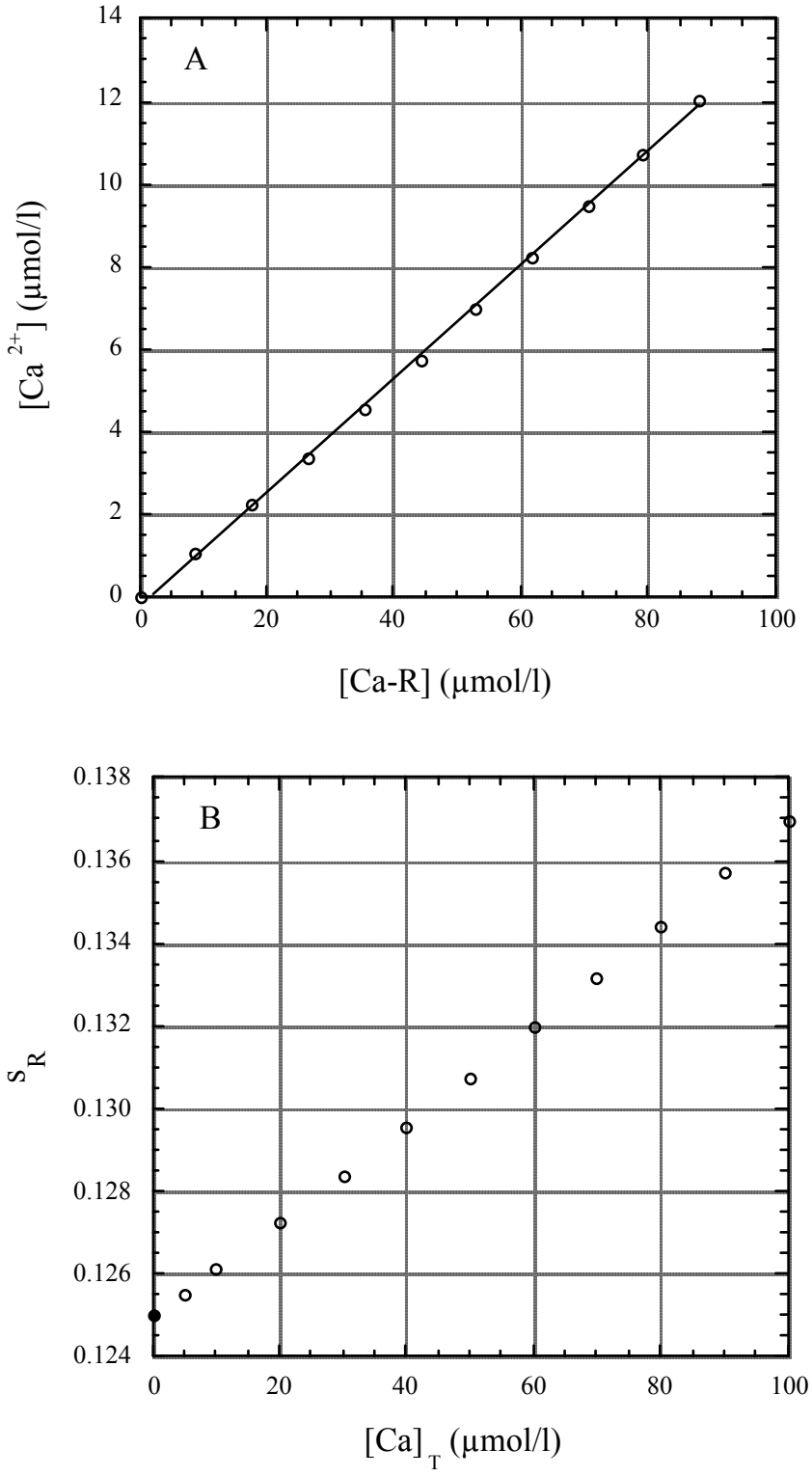

Figure 2. A. Estimation of s_R with a $[Ca]_T$ of 100 µmol/l. The linear regression draw through the points was:

$[Ca^{2+}] = - 0.1631112 + 0.136979*[Ca\text{-}R]$; R = 0.9996696

B. Plot of the calculated values of s_R for each value of $[Ca]_T$. The filled circle at zero $[Ca]_T$ is the true value of 0.125.

The linear regression gave a value of s_R of 0.13698 mmol/l. Moreover, as shown in Figure 3, the value calculated for s_R is almost a linear function of $[Ca]_T$. As the $[Ca]_T$ decreases, s_R tends to 0.125, but even at a $[Ca]_T$ of 5 μmol/l, it is overestimated. s_R is also tabulated in Table 1.

Step 2: Estimate [Ca-R], [Ca^{2+}] and [Ca-EGTA]

1) Estimate the concentration of [Ca-R]: In an experiment the resin would be spun down and [Ca-R] estimated from equation [4] namely:

$$[Ca-R] = [Ca]_T * \frac{100\,\%Ca^{45} - \%Ca^{45}(sup\ erna\ tan\ t)}{100\,\%Ca^{45}} \qquad [4]$$

The assumption was made that in this estimation, the error would be minimal and the measured concentration would be similar to the true concentration. To mimic this, the actual [Ca^{2+}] was calculated from equation [33] This cubic equation was solved using the Newton Raphson method. Knowing the [Ca^{2+}], the [Ca-R] was estimated from equation [6] namely:

$$[Ca-R] = \frac{[R]_T[Ca^{21}]}{([Ca^{2+}] + K_R)}$$

2) Estimation of [Ca^{2+}]: In an actual experiment [Ca^{2+}] is estimated from the measured [Ca-R] as follows:

   $[Ca^{2+}] = s_R * [Ca\text{-}R]$

   At each $[Ca]_T$, [Ca^{2+}] was estimated from the measured s_R

3) From the estimated [Ca-R] and [Ca^{2+}] the [Ca-EGTA] can be calculated from equation [14]:

$$[Ca-EGTA^{2-}] = [Ca]_T - ([Ca^{2+}] + [Ca-R])$$

Step 3: Estimate K_{app} and purity from a Scatchard plot

Having calculated both [Ca^{2+}] and [Ca-EGTA], [Ca-EGTA] (x-axis) is plotted against $\frac{[Ca-EGTA]}{[Ca^{2+}]}$. This should give a straight line, with the intercept on the x-axis giving the purity and the slope the K_{app}. In Figure 3A the Scatchard plots for 100 μmol/l, 50 μmol/l, 20 μmol/l and 5 μmol/l $[Ca]_T$ are illustrated and all the results are tabulated in Table 1. As $[Ca]_T$ is reduced the ratio Bound/Free increases. This arises because s_R is a function of the $[Ca]_T$, increasing as $[Ca]_T$ increases and because of this [Ca^{2+}] is overestimated. The pK_{app} values as shown

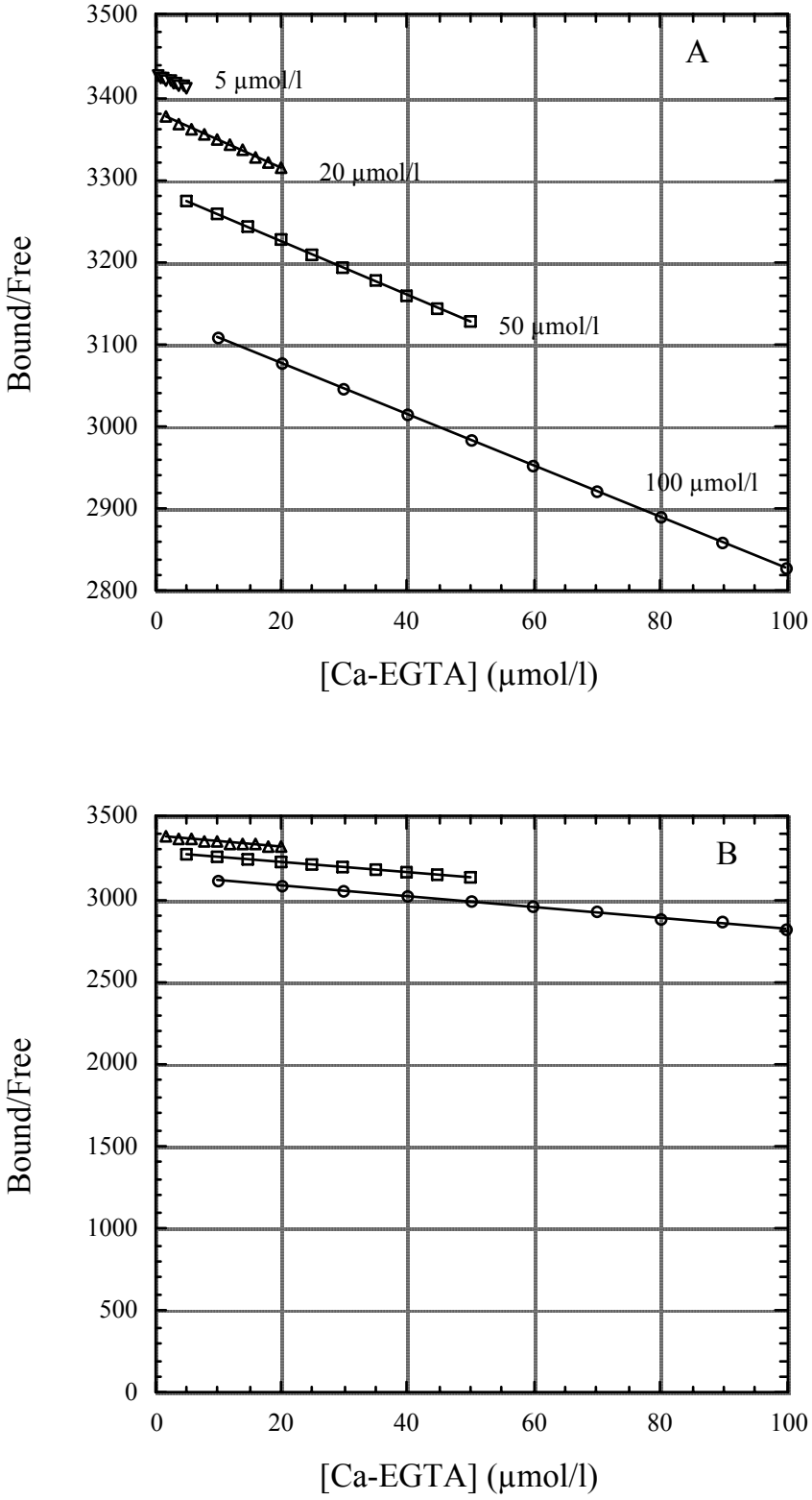

Figure 3: A. Scatchard plots of the results from $[Ca]_T$ of 100 µmol/l, 50 µmol/l, 20 mmol/l and 5 µmol/l. The regression coefficient in each case was 1.00000000. For further details see text and Table 1. B. Same Scatchard plots as in A, but with the y-axis starting at zero. This emphasises the narrow range of the measurements. For clarity the plot for 5 µmol/l has been omitted.

Table 1: Results of Chelex-Partition method

$[Ca]_T$ (μmol/l)	s_R	pK_{app}	$[EGTA]_T$ (mmol/l)
100	0.136979	6.49625	1.002270
90	0.135698	6.50033	1.002276
80	0.134437	6.50439	1.002284
70	0.133194	6.50842	1.002288
60	0.131971	6.51242	1.002296
50	0.130765	6.51641	1.002304
40	0.129578	6.52303	1.002306
30	0.128407	6.52431	1.002313
20	0.127255	6.52822	1.002323
10	0.126119	6.53211	1.002323
5	0.125557	6.53405	1.002337

in Table 1 increased from 6.49625 at a $[Ca]_T$ of 100 μmol/l to 6.53405 at 5 mmol/l. Even at this concentration the method did not correctly predict the pK_{app} value. In the Scatchard plot moreover, as the $[Ca]_T$ is decreased the range of Bound/Free is decreased and this illustrated in 3B where the Bound/Free axis starts at zero. Purity was estimated from the Scatchard plot (see Table 1) but the linear fit has to be extrapolated from at the best, 100 μmol/l to 1 mol/l. Because of this not much weight can be put on such estimations. This Figure also illustrates the narrow range of calcium concentrations over which pK_{app} is being estimated.

Step 4: Estimate the $[Ca^{2+}]$ in the buffer solutions

This was calculated on the assumption of $[EGTA]_T$ of 3.8 mmol/l and for the pK_{app} values estimated with a $[Ca]_T$ of 100 μmol/l, 50 μmol/l, 20 μmol/l and 5 μmol/l. The results are shown in Figure 4. Despite the variation in pK_{app}, the actual differences in the $[Ca^{2+}]$ are minimal, the maximum difference being 0.5 μmol/l for solution 10, with pK_{app} estimated with 100 μmol/l $[Ca]_T$.

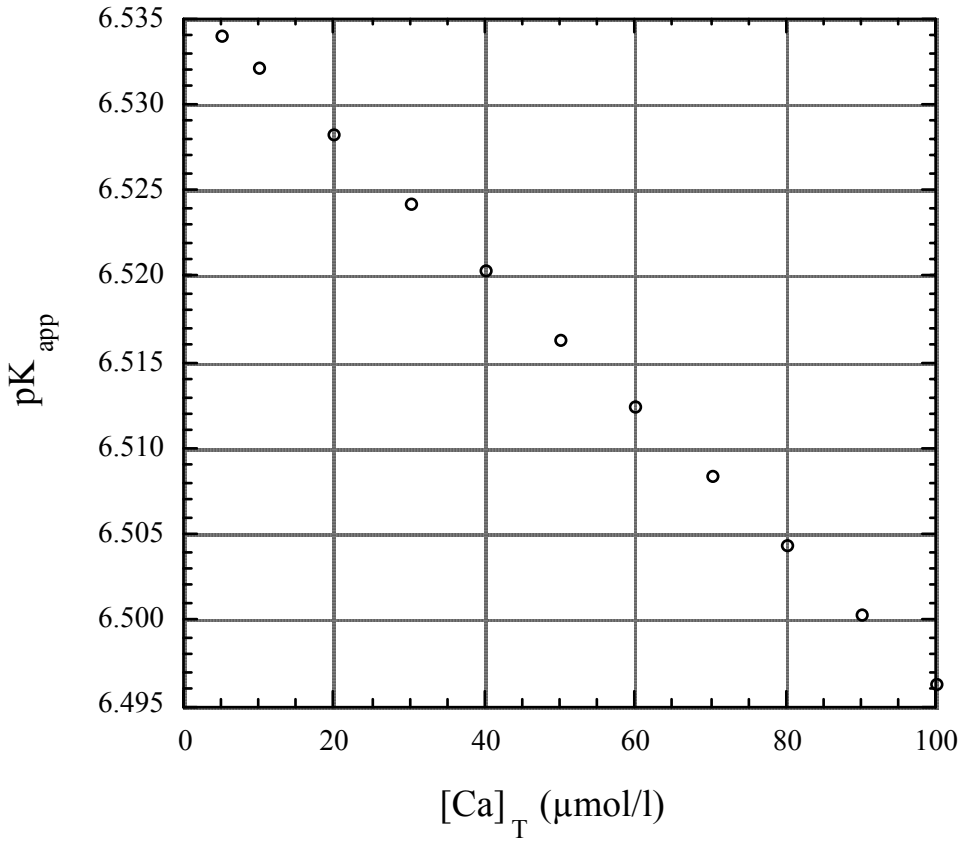

Figure 4: Calcium concentrations in the buffer solutions 1 to 10, calculated using the estimated pK_{app} values for, $[Ca]_T$ of 20 µmol/l (open diamonds), $[Ca]_T$ of 50 µmol/l (open squares) and $[Ca]_T$ of 100 µmol/l (open circles). The filled circles represent the actual $[Ca^{2+}]$ concentrations

CRITIQUE OF THE METHOD

There are three major disadvantages with the method, namely

1) The $[Ca]_T$ have to be in the µmolar range in order that the relationship between $[Ca^{2+}]$ and [Ca-R] is approximately linear.
2) It takes time to reach equilibrium between resin and Ca^{2+}
3) Even with an ideal data set the answer is not correct.

1) means that great care has to be taken to minimise the contaminating calcium concentrations. This can be done, but is technically difficult and limits the use of the method. The relationship between $[Ca^{2+}]$ and [Ca-R] is only approximately linear and even at a $[Ca]_T$ of 5 µmol/l the predicted K_R was 0.1255575 instead of 0.125. To be realistic $[Ca]_T$ must be between 50 µmol/l and 100 µmol/l and in this range pK_{app} is lower than the true pK_{app} and the calculated $[Ca^{2+}]$ slightly larger than the true $[Ca^{2+}]$.

2) This comes from R. E. Godt himself. His results are wrong because equilibrium was not reached between the resin and Ca^{2+}. In any future studies it would have to be shown that equilibrium was indeed reached.
3) In the calculations an ideal data set was used for the calculations. Despite this the calculated pK_{app} values were not correct.

It is a very indirect method, tedious and time consuming. It is dated and is now only of historical interest.