IN VIVO AND IN VITRO STUDIES
ON IONIZED VERSUS TOTAL SERUM CALCIUM
IN HYPERPARATHYROIDISM

By
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ABSTRACT
Measurements of ionized (Ca++ ) calcium and total (Ca_t) calcium have been performed in sera from hyperparathyroid, parathyroidectomized and control subjects. The relationship between Ca++ and Ca_t has been analysed statistically. It was found that two straight lines with the point of intersection at Ca++ = 2.75 and Ca_t = 5.55 mEq/l, and slopes of 0.628 and 0.447, respectively, would describe the data more accurately than one single line. However, when the calcium level was varied by the addition of calcium chloride in vitro in individual or pooled sera, the Ca++-Ca_t relationship appeared to be linear. The results suggest that sera from hyperparathyroid subjects have an increased calcium binding capacity.

Serum calcium consists of three different fractions. Free calcium ions (ionized calcium, Ca++ ), as well as total serum calcium (Ca_t), can be measured directly. About 10 per cent of Ca_t is neither ionized nor protein bound, but occurs as complexes with citrate, lactate, sulphate, bicarbonate and other serum anions. The complex-bound calcium fraction can be determined as the difference between ultrafilterable calcium and ionized calcium. It is generally believed that determination of complex-bound calcium is of little clinical interest except in patients with a reduced glomerular filtration rate.

Ionized calcium, the physiologically active fraction of serum calcium, amounts
to about 46 per cent of Ca in normal subjects, according to measurements with a calcium-specific ion-selective electrode (Lindgärde 1972).

Of protein-bound calcium, 80–90 per cent is an albumin chelate (Moore 1969; Pedersen 1971). Variations in serum albumin concentration, particularly lowered albumin frequently encountered in disease, therefore lessen the clinical value of total calcium determinations. As the relation between free and protein-bound calcium is governed by the law of mass action, total serum calcium can be adjusted by reference to the albumin concentration in the sample. This procedure yields a narrower "normal range", allowing easier detection of pathological deviations (Orrell 1971).

The interest in direct determination of serum Ca\(^{++}\) has been stimulated ever since the suggestion of Lloyd & Rose (1958) that in hyperparathyroidism the ability of plasma proteins to bind calcium may be reduced. This hypothesis has been the object of considerable debate, as conflicting results have been presented (Lloyd et al. 1962; Walser 1962; Dale & Kellerman 1967; Transböl et al. 1970; Wills & Lewin 1971; Pedersen 1972). The object of the present report is to contribute to the understanding of the relations between Ca\(^{++}\) and Ca\(_t\) by a statistical analysis of determinations performed in sera from controls as well as from hyperparathyroid and parathyroidectomized patients.

**MATERIAL**

The determinations were performed during the period December 1970 – August 1972. The three groups of subjects studied were:

*Group 1.* – Control cases were 41 hospitalized patients with normal renal function and a serum Ca\(_t\) ranging between 4.5 and 5.2 mEq./l. The Ca\(^{++}\) range was 1.98–2.54 mEq./l (mean ± 2 sd).

*Group 2.* – 27 patients with primary hyperparathyroidism. A total of 86 determinations of Ca\(^{++}\) and Ca\(_t\) were performed.

*Group 3.* – Five of the group 2 cases were studied shortly after parathyroidectomy, at a time when the Ca\(^{++}\) was subnormal (below –2 sd of group 1).

**EXPERIMENTAL METHODS**

Venous blood was drawn from fasting subjects in the morning. Serum calcium ion activity (Ca\(^{++}\)) was measured potentiometrically with a calcium ion sensitive electrode system connected to a digital pH meter (Orion Research Corp., Cambridge, Mass. Calcium selective flow through electrode No. 99–20: pH meter model 801). Details of the method have been presented previously (Lindgärde 1972). The serum pH was determined by the use of a blood microsystem (Type BMS 1b, Radiometer, Copenhagen) at 37°C immediately after the Ca\(^{++}\) measurement.

The total serum calcium (Ca\(_t\)) measurements were made in a flame photometer (Eppendorf), or in an atomic absorption spectrometer (Perkin-Elmer).
The total serum protein was estimated by a biuret method. Serum albumin was determined by the bromocresol method of (Northam & Widdowson 1967) in 22 control patients and in the sera from 15 hypercalcaemic patients. The corresponding Ca₄ was estimated by atomic absorption spectrometry.

**In vitro addition of calcium**

The above measurements were supplemented by Ca₄ and Ca⁺⁺ determinations after the addition of Ca⁺⁺ *in vitro*. Eight sera were used for this purpose. Each serum sample was divided into several aliquots. To each 2 ml aliquot a total of 200 µl of CaCl₂ and NaCl solutions was added. Different Ca₄ concentrations were obtained by the use of various volumes of solutions with varying CaCl₂ concentrations. Constant volume of addition was maintained with 0.15 m NaCl.

**Statistical methods**

The fit of various regression curves to points in the Ca⁺⁺/Ca₄ diagram was tested statistically. Briefly, two types of regression curves were tested, i.e. (1) a straight line and (2) a combination of two straight lines connected in one point (see Fig. 2). For the combination of straight lines (2) the minimum of the sum of squares of the deviations from the empirical Ca⁺⁺ values was calculated by computer. The linear regression equation (1) was computed using standard statistical formulas.

**RESULTS**

**In vitro experiments**

When increasing amounts of calcium were added to sera the Ca⁺⁺ values were linearly related to the Ca₄ values. The correlation coefficients were 0.99–1.0 except in one case (Table 1). The slope of the line, dCa⁺⁺/dCa₄, varied but was above 0.50 in all experiments, indicating a greater fractional increase of Ca⁺⁺ than of Ca₄. Obviously there was little influence of pH levels on the slope.

The patient A. L. is a case of myelomatosis with increased Ca₄ and normal ionized calcium which could be explained by the presence of a calcium binding myeloma globulin (Lindgärde & Zettervall 1973). This serum gave a slope of 0.54 when the total protein concentration was 9.5 g/100 ml. When the serum protein concentration in sample E. S. was diluted from 7.0 g/100 ml (alb. 4.8 g/100 ml) to 6.0 g/100 ml (alb. 4.1 g/100 ml) the slope increased from 0.63 to 0.65. This indicates that the slope is not influenced significantly by the protein concentration in the range examined.

**In vivo**

*pH correction of Ca⁺⁺*. – The average pH values in the hyperparathyreoid, control and parathyroidectomized groups were 7.377 ± 0.061, 7.382 ± 0.050 and 7.365 ± 0.056 respectively. On two occasions in the hyperparathyreoid
Table 1.
Ionized and total serum calcium values after addition of calcium chloride to sera in vitro. Linear regression analysis.

<table>
<thead>
<tr>
<th>Diagnos</th>
<th>pH</th>
<th>Slope of the regression line</th>
<th>Correlation coefficient</th>
<th>Total serum calcium mEq./l</th>
<th>Ionized serum calcium mEq./l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pooled sera</td>
<td>7.49</td>
<td>0.64</td>
<td>0.998</td>
<td>4.4-7.7</td>
<td>1.84-4.00</td>
</tr>
<tr>
<td>Blood donors</td>
<td>7.20</td>
<td>0.59</td>
<td>0.997</td>
<td>4.4-8.0</td>
<td>2.54-4.72</td>
</tr>
<tr>
<td>Pooled sera</td>
<td>7.40</td>
<td>0.61</td>
<td>0.999</td>
<td>4.5-8.1</td>
<td>2.02-4.24</td>
</tr>
<tr>
<td>Blood donors</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F. L. normal</td>
<td>7.42</td>
<td>0.52</td>
<td>0.999</td>
<td>4.3-8.0</td>
<td>1.92-3.84</td>
</tr>
<tr>
<td>E. S. normal</td>
<td>7.38</td>
<td>0.56</td>
<td>0.997</td>
<td>4.3-7.9</td>
<td>2.05-4.05</td>
</tr>
<tr>
<td>A. L. Myelomatosis</td>
<td>7.40</td>
<td>0.54</td>
<td>0.995</td>
<td>4.3-8.3</td>
<td>2.08-4.55</td>
</tr>
<tr>
<td>P. R. Nephrolithiasis</td>
<td>7.38</td>
<td>0.50</td>
<td>0.964</td>
<td>4.9-9.4</td>
<td>2.21-4.49</td>
</tr>
<tr>
<td>F. H. Phosphate treated hypercalcaemia</td>
<td>7.38</td>
<td>0.62</td>
<td>0.993</td>
<td>4.9-9.1</td>
<td>2.41-4.92</td>
</tr>
<tr>
<td>H. A. Hyperparathyroidism</td>
<td>7.45</td>
<td>0.68</td>
<td>0.989</td>
<td>6.2-9.9</td>
<td>2.60-5.32</td>
</tr>
</tbody>
</table>

group the pH values were below 7.30. There was no obvious tendency for the higher calcium values to be combined with lower pH values (linear regression analysis). In order to avoid the influence of different pH values, each individual Ca\(^{++}\) value was corrected according to the formula:

\[
\log (\text{Ca}^{++}) \text{ at pH 7.38} = \log (\text{Ca}^{++}) \text{ at pH measured} + 0.297 (\text{pH -7.38})
\]

(Lindgärde 1972).

**Serum protein concentration**

The total serum calcium is essentially a function of the ionized calcium level and the protein concentration. In some control and hypercalcaemic patients the serum albumin was measured instead of the total serum protein. The average values are given in Table 2.

In order to evaluate the influence of protein concentration on the distribution of Ca\(^{++}\) and Ca\(_t\) values in the hyperparathyreoid group the calcium levels in the samples with a low protein concentration, i. e. total serum protein or albumin below 6.9 or 4.1 g/100 ml, respectively, are indicated in Fig. 1. No striking difference between the groups was apparent. In the hyperparathyroid group there was no correlation between total serum protein or albumin concentration and the Ca\(_t\) (linear regression analysis). In the hypocalcaemic samples drawn
Table 2.
Protein concentration in hyperparathyroid, control and parathyroidectomized patients.
The $P$ values for between-group differences are indicated.

<table>
<thead>
<tr>
<th>Group</th>
<th>Total serum protein g/100 ml</th>
<th>Albumin g/100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.12 ± 0.51</td>
<td>3.74 ± 0.63</td>
</tr>
<tr>
<td>Hyperparathyroid</td>
<td>6.92 ± 0.61</td>
<td>4.15 ± 0.41</td>
</tr>
<tr>
<td>Parathyroidectomized</td>
<td>6.35 ± 0.57</td>
<td>-</td>
</tr>
</tbody>
</table>

in the postoperative state the average total serum protein concentration was significantly depressed.

Average Ca$^{++}$ and Ca$_t$ in the control group

The average Ca$_t$ and pH corrected Ca$^{++}$ were 4.81 ± 0.20 and 2.26 ± 0.14 mEq./l respectively. Those of the 19 sera in which Ca$_t$ was estimated by flame photometry gave a mean of 4.86 ± 0.19 mEq./l with a corresponding Ca$^{++}$ 2.30 ± 0.10. The Ca$_t$ values measured with atomic absorption spectrometry gave a mean of 4.76 ± 0.20 mEq./l and 2.22 ± 0.16 for Ca$^{++}$. The mean

![Graph](image_url)

Fig. 1.
Total serum calcium (Ca$_t$) and ionized serum calcium (Ca$^{++}$) in controls (O) and hyperparathyroidism (●) with regression lines. Values from sera with low protein concentration are indicated with x.

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ratio Ca\(^{++}/Ca\(_i\) in these groups were 0.474 ± 0.019 and 0.467 ± 0.040. There were no statistical differences between the Ca\(^{++}\) or Ca\(_i\) values in the two groups (0.05 < \(P\)).

**Distribution of serum calcium values in hyperparathyroidism compared to the control group**

The observations in the hyperparathyroid patients are given in Fig. 1 together with the control group. The regression equation on Ca\(^{++}\) for Ca\(_i\) is: \(y = 0.16 \pm 0.469 \times X\). Ca\(^{++}\) and Ca\(_i\) are intercorrelated with \(r = 0.85\) (\(P < 0.001\)). In order to evaluate whether the ionized calcium fraction was increased more than Ca\(_i\) in the hyperparathyroid group a covariance analysis was performed between the hyperparathyroid and control groups. For any given value of Ca\(_i\), Ca\(^{++}\) was significantly higher in the hyperparathyroid group.

**Evaluation of the distribution of Ca\(^{++}\) and Ca\(_i\) in hyperparathyroid, control and parathyroidectomized groups**

Fig. 2 shows the Ca\(^{++}\) and Ca\(_i\) data in all three groups. Using the criterion of best fit as given in Statistical Methods the best combination of two straight lines found was that with the point of intersection in Ca\(_i\) = 5.55, Ca\(^{++}\) = 2.75 mEq./l, and slopes 0.628 and 0.447 respectively.

The sum of squares of ordinate deviations was 6.169. This is lower than the corresponding parameter of the straight regression line, 6.492 (\(P = 0.008\)). If
the sera from the parathyroidectomized patients are excluded from the material
the difference is still significant \( P = 0.042 \) with the slopes being 0.626 and
0.446. If the data in which \( \text{Ca}_t \) was estimated by atomic absorption spectrometry
(22 observations in the control group and 15 in the hyperparathyroid group)
are excluded, the fit of the two-line regression is still better than that of the
single regression line \( P = 0.0012 \), and the slopes of the two lines are 0.669
and 0.434.

**DISCUSSION**

By means of various methods it is possible to estimate all three calcium fractions
in the serum, viz. the ionized, the complexed, and the protein bound calcium.
In this study ionized serum calcium and total serum calcium have been measured.
There are no reports indicating that determination of complex-bound ultrafilterable calcium yields any valuable information with regard to hyperparathyroidism. Several attempts have been made to estimate the calcium-protein dissociation constant in hyperparathyroidism. The data available do
not indicate any deviation from the normal in this regard. However, possible changes in serum calcium distribution in hyperparathyroidism may not be demonstrable by ultrafiltration, as this procedure may disturb calcium protein interactions. Determination of serum ionized calcium by means of an ion-specific electrode is more likely to reflect the *in vivo* situation.

The aim of the present study was to evaluate the usefulness of \( \text{Ca}^{++} \) determination in borderline hypercalcaemia and to study the relationship between \( \text{Ca}^{++} \) and \( \text{Ca}_t \) in hyperparathyroidism. The relationship between free and protein-bound calcium is influenced by the pH, protein concentration, and temperature. Therefore the individual \( \text{Ca}^{++} \) values were corrected to pH 7.38, the average pH of the groups, applying an empirical correction formula. The protein concentration in the operated hypocalcaemic group was significantly lower than in the control group and a few samples in the hyperparathyroid group where albumin was measured, gave a higher average than in the control group. However, when those calcium values associated with low protein or high albumin concentration (Table 2) were excluded, no change in the statistical results was obtained.

As only fresh sera have been used for the \( \text{Ca}^{++} \) and \( \text{Ca}_t \) analysis there might have been methodological variations during the period of sampling. However, during the same time period no drift in analytical results was apparent in the control material. In 132 unselected adult patients Pittinger et al. (1971) found a \( \text{Ca}^{++} \) average of 2.16 ± 0.12 mEq./l. In a previously published population study (Lindgärde 1972) the mean \( \text{Ca}^{++} \) level was 2.23 ± 0.14 mEq./l. The average pH in that study was 7.38, i.e. the same as in the present control groups. Therefore this control group with a mean of 2.26 ± 0.14 mEq./l does
not seem to appreciably deviate from an apparently healthy control group with regard to Ca\(^{++}\).

When calcium is added to serum at constant pH and protein concentrations a regular increase in the ratio Ca\(^{++}\)/Ca\(_t\) appears. This finding is consistent with the results of Hansen & Theodorsen (1971). In some of the experiments with addition of CaCl\(_2\) the Ca\(^{++}\) determinations were checked 24 h later and found to be identical with the values observed immediately after the addition. This indicates that the calcium-protein interaction equilibrium was immediately attained.

Using the nomogram, constructed by McLean & Hastings (1935), and applying linear regression analysis, the slope of the regression line for Ca\(^{++}\) on Ca\(_t\) in the range 4.0–8.0 mEq./l and protein concentration 7.0 g/100 ml is estimated as 0.50. This result is to be compared with the single line regression equation from the in vivo results in this study giving a slope of 0.53. However, the findings of the co-variance analysis of the control group and the hyperparathyroid group suggest that the control values fall outside the regression line for the data of the hyperparathyroid group. Hence the relationship between Ca\(^{++}\) and Ca\(_t\) in vitro may be better described by curvilinear rather than linear regression. Confirming this it was found that a regression curve consisting of two straight lines connected at one point would describe the data more accurately than one single line. The intersection point was found to be a Ca\(_t\) = 5.55 mEq./l. The two straight lines with different slopes may explain the conflicting results about the usefulness of Ca\(^{++}\) determination in hypercalcaemia in earlier reports.

When the Ca\(_t\) of individual sera is varied, at constant protein concentration, by the addition of calcium chloride, the Ca\(^{++}\)–Ca\(_t\) relationship appears linear with correlation coefficients of 0.96–1.0. The slope and the position of the regression line differs between the individual sera. This does not necessarily mean, that points in a Ca\(^{++}\)–Ca\(_t\) diagram obtained with randomly chosen Ca\(_t\) and the corresponding Ca\(^{++}\) from a large collection of randomly chosen normal sera in vitro will correlate linearly. The type of regression obtained evidently depends on the statistical distribution of the slopes and axis intercepts of the in vitro regression lines for normal sera. Thus the finding of an apparent curvilinear Ca\(^{++}\)–Ca\(_t\) regression in vivo using single Ca\(^{++}\) and Ca\(_t\) determinations on individual sera does not necessarily imply that sera from hyperparathyroid patients contain more calcium ligands than normal sera. However, the slopes of the regression lines obtained with in vitro data all exceed the slope of the regression line for the in vivo material; and the same applies to the slopes of the regression lines from the in vitro experiments with the two pooled sera. These results suggest that sera from hyperparathyroid patients have indeed an increased calcium binding capacity.

It is obvious from the present data that Ca\(^{++}\) determinations in borderline
hypercalcaemia may be of value in some patients. In general, Ca\(^{++}\) seems to be increased more than Ca\(_t\) in vivo up to a Ca\(_t\) value of 5.55 mEq./l. Therefore repeated Ca\(^{++}\) determinations may be more effective for disclosing a borderline hypercalcaemia than the corresponding Ca\(_t\) measurements.

Moore (1969) found in cancer patients with hypercalcaemia that the calculated percentage for calcium albuminate was lower than in normals while calcium globulinate accounted for 33.5 per cent of the calcium proteinate, which is significantly higher than in normal subjects. Hence the finding of an increased bound calcium in this study may be interpreted in terms of variation in unknown plasma proteins with high calcium affinity. The newly discovered alpha\(_1\) globulin with high calcium binding capacity (Haupt et al. 1972), and the not yet identified serum components in alpha\(_2\) and beta regions with apparently firm calcium binding capacities (Lindgärde et al. 1973), may be involved in this process.

It is interesting to speculate on an in vivo homeostatic mechanism that functions just above the physiological Ca\(^{++}\) level. In monkeys Raman (1971) found that low doses of calcitonin reduced only total serum calcium whereas high doses depressed both total and ionized calcium levels. Similar results have been reported by Binderman et al. (1972). They found that small doses of calcitonin caused a significant fall in total calcium in rat plasma without changes in concentration of dialysable calcium. When ten times larger dosages were given to rats, Gitelman et al. (1965) found a decrease in both ionized and total calcium. Therefore it may be assumed that calcitonin reduces the protein bound calcium fraction but that as the Ca\(^{++}\) level falls the immediately increased parathyroid hormone level will counteract this effect. However, it is difficult to compare acute experimental conditions in animals with the conditions in human disease. Tashijan et al. (1970) determined serum calcitonin in patients with chronic hypercalcaemia. Surprisingly, most of the subjects had normal calcitonin levels. Therefore a possible PTH-mediated increased calcium binding to protein may not always be counteracted by calcitonin.

ACKNOWLEDGMENT

The study was supported by a grant from the Alfred Usterlund Foundation.

REFERENCES


Received on March 13th, 1973.