A BIOLOGICAL METHOD FOR THE ESTIMATION OF CALCIUM ION CONCENTRATION

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The sensitivity of the frog’s heart to changes in the calcium content of its nutrient fluid has long been known. Straub (1) commented that this sensitivity could be made use of to titrate calcium concentrations. Trendelenburg and Goebel (2) used it to demonstrate the reduction in calcium in the serum of the cat in parathyroid tetany. Klinke (3), in his comprehensive review, mentions the frog’s heart as a possible biological indicator of calcium ion concentrations, but expresses doubt, based partly on the work of Günther and Heubner (4), as to whether its contraction is affected by calcium in the ionized form alone. The use made of the frog’s heart by Clark, Percival, and Stewart (5) in a study of calcium and citrate mixtures is referred to in detail in the accompanying paper (6).

The method here described is the result of an attempt to utilize this sensitivity of the frog’s heart, in a strictly quantitative manner, for the estimation of calcium ion concentrations in certain saline solutions and biological fluids. The validity of the method is based upon the following assumptions or demonstrations:

1. That all of the calcium in the solutions used as standards of reference is present in ionized form, even when these solutions are supersaturated with respect to CaCO₃. Since all Ca⁺⁺ observations are expressed in terms of the standard solutions, any error due to this assumption would have a corresponding effect upon the Ca⁺⁺ concentrations estimated by the method.

* A preliminary communication has been published previously (Proc. Soc. Exp. Biol. and Med., 30, 1344 (1932–33)).

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2. That the frog's heart is sensitive to changes in the concentration of Ca++, but not to changes in the concentration of calcium in non-ionized form. That this is true when calcium is combined with citrate is shown in the accompanying paper (6). That it is true when calcium is combined with protein has also been shown (7).

3. That, other conditions being identical, two solutions inducing an identical response from the frog's heart, in a range of Ca++ concentration in which the heart is able to respond to changes in either direction, have an identical concentration of Ca++.

4. That in the case of biological fluids, it being impossible to make the solutions of reference exactly comparable, the results may be regarded as valid, provided certain precautions, to be described, are observed.

**Method**

The method consists essentially in the direct comparison and matching of known solutions with solutions or biological fluids of unknown Ca++ concentration, the criterion for equal Ca++ concentration being equal amplitude of contraction of the ventricle of the isolated heart of the frog, as recorded on a drum. It is emphasized that the method depends upon direct matching and does not involve calculations from one set of conditions to another. Provided, therefore, that the heart used is sensitive to changes within the range in which Ca++ is present, a condition which must in each instance be demonstrated, the method is independent of the particular state of activity, or particular amplitude of contraction, of the particular heart used as an indicator.

**Preparation**—The preparation is the isolated heart of the frog, suspended from a modified Straub cannula and so arranged that the amplitude of contraction of the ventricle will be recorded, greatly amplified, upon a moving drum.

Healthy, full-blooded frogs of medium size are chosen. Frogs showing edema of the legs are particularly to be avoided. A frog is pithed and the heart exposed. A straight cannula (Fig. 1) 5.0 to 5.5 cm. long over-all, with an internal diameter of 6.0 mm., a capacity of about 1.0 cc., and a tip with an outside diameter not to exceed 2 mm., is inserted through the left branch of the aorta into the ventricle, where it is held by a ligature around the aorta.
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The heart is raised from the body, a second ligature is passed around all the tissues to which the heart is attached, care being taken not to injure the auricles, and the attachments are severed beyond the ligature. The cannula is filled with a suitable fluid, and the fluid repeatedly changed until all blood has been washed out. For changing the fluid a medicine dropper with a tip long enough to reach the constricted portion of the cannula is used.

If the preparation has been properly made, the fluid in the cannula will move with an excursion of several mm. with each beat of the heart, and there will be no escape of fluid. If fluid escapes, indicating damage to the preparation, a new heart will usually be required. The chief source of difficulty in making the preparations is in reaching the cavity of the ventricle, and in securing the cannula in place so that communication is unobstructed. Some difficulty may be experienced in passing through the aortic valve, and it is important not to use force, the best results being obtained by teasing the valve orifice over the tip of the cannula.

A silk thread is either tied around the tip of the ventricle or to a small clip attached to the apex. The former procedure is most easily carried out with the heart and cannula empty, and with the cannula inserted through a rubber stopper held firmly by a clamp. The preparation is suspended in a simple chamber, made

![Fig. 1. Modified Straub cannula](Image)
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from the upper 7 cm. of a Pyrex test-tube of 22 mm. diameter, with a tightly fitting rubber stopper, the chamber being kept moist with a piece of moistened filter paper adhering by capillarity to the posterior wall. The thread is passed through pulleys to a writing lever. The recording system should be as nearly free from friction and inertia as possible, and with these precautions, and proper counterbalancing of the lever, the amplitude of excursion of the writing point on the drum may be adjusted at will. An excursion of about 8 to 9 cm. for the maximum amplitude of contraction gives the desired sensitivity to the method. During the period while adjustments are being made the fluid in the cannula should be changed frequently.

A suitable preparation may under favorable conditions be used over a period of 2 to 4 days. While the preparation is at room temperature, the fluid should be changed frequently, but it may be left in the ice box overnight without change of fluid, and if still usable will recover promptly on being brought to room temperature and on change of fluid. Any preparation which has been exposed to solutions or fluids toxic to the heart will, of course, have a shorter period of usefulness.

Solutions of Reference—The requirements of the solutions of reference are: (1) they must be capable of sustaining the action of the heart; (2) their Ca++ concentration must be accurately known; (3) they must approach as nearly as possible the composition of the unknown solutions or biological fluids with which they are to be matched.

Our earlier observations on biological fluids were made by analyzing each fluid separately for its inorganic constituents, the solutions of reference being made up on the basis of these analyses. As a result of investigations reported in this paper, we have found it possible, in making routine observations, to use two standard series of solutions of reference, one especially adapted to observations upon serum and the other to observations upon cerebrospinal fluid. These differ from one another only in their potassium content, and are based upon average values for the inorganic constituents of normal fluids. It appears that under ordinary conditions the use of these standard solutions of reference does not introduce an error greater than 0.1 mM per liter of Ca++ in individual determinations, but it should be pointed out that this error
may be exceeded in the case of fluids with marked deviation from the normal in electrolyte content, and that under such conditions, particularly when the potassium content is high, actual analysis of the unknown fluids may be desirable.

The standard solutions of reference are based upon the solution of van Dyke and Hastings (8). They differ from Ringer's and Locke's solutions chiefly in that they are buffered with approximately the NaHCO₃ concentration of human serum, and that the pH is controlled and the solutions are oxygenated by saturation with an appropriate CO₂-O₂ mixture. They are isotonic with, and have approximately the ionic strength of mammalian fluids. Phenol red, which does not affect the heart in the concentrations

<table>
<thead>
<tr>
<th>CO₂ per cent</th>
<th>NaHCO₃ mm per l.</th>
<th>154 mm NaHCO₃ cc. per l.</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5</td>
<td>19.2</td>
<td>124.6</td>
</tr>
<tr>
<td>4.0</td>
<td>21.9</td>
<td>142.2</td>
</tr>
<tr>
<td>4.5</td>
<td>24.8</td>
<td>161.0</td>
</tr>
<tr>
<td>5.0</td>
<td>27.4</td>
<td>178.0</td>
</tr>
<tr>
<td>5.5</td>
<td>30.1</td>
<td>195.5</td>
</tr>
<tr>
<td>6.0</td>
<td>32.9</td>
<td>213.7</td>
</tr>
</tbody>
</table>

used, serves as a constant check on pH. Nearly all of the solutions are supersaturated with respect to CaCO₃, but unless allowed to become too alkaline they will usually be serviceable for 1 day. Any solutions from which precipitation of CaCO₃ has begun must be discarded.

Preparation of Solutions of Reference—For routine observations it is convenient to keep in the laboratory basic solutions containing all of the constituents of the solutions of reference except calcium, which must be added shortly before the solutions are to be used. The basic solutions are kept saturated (at room temperature) with a mixture of approximately 4.0 per cent CO₂ in O₂. The concentration of NaHCO₃ in these solutions should be adjusted so that when saturated at 25° with the CO₂-O₂ mixture the pH at 38° would be between 7.35 and 7.40, in order that when observations are
made at room temperature the pH of the fluid and of the solutions of reference will be comparable. Mixtures of CO₂ and O₂ may be obtained on specification from commercial sources, but calculations should be based upon accurate analyses. Table I may be used to facilitate calculation of the NaHCO₃ concentration appropriate to the gas mixture used.

The stock and basic solutions may be made up as indicated in Table II, and the basic solutions saturated at room temperature with the CO₂-O₂ mixture. Saturation should be repeated whenever solutions of reference are to be made.

**Table II**

*Composition of Stock Solutions Isotonic with Each Other and with Mammalian Fluids, and of Basic Solutions, Assuming Gas Mixture of 4.0 Per Cent* *CO₂* *in* O₂

<table>
<thead>
<tr>
<th>Salt</th>
<th>Stock solutions</th>
<th>Basic solutions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mm per l.</td>
<td>gm per l.</td>
</tr>
<tr>
<td>NaCl (anhydrous)</td>
<td>154.0</td>
<td>9.00</td>
</tr>
<tr>
<td>KCl</td>
<td>154.0</td>
<td>11.48</td>
</tr>
<tr>
<td>NaHCO₃ (anhydrous)</td>
<td>154.0</td>
<td>12.93</td>
</tr>
<tr>
<td>MgCl₂·6H₂O</td>
<td>102.7</td>
<td>20.88</td>
</tr>
<tr>
<td>Phenol red</td>
<td></td>
<td>1.00</td>
</tr>
<tr>
<td>Glucose</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* For gas mixtures other than 4.0 per cent CO₂, see Table I.
† This concentration is appropriate to cerebrospinal fluid. We have generally used 5.0 mm per liter, or 32.5 cc. of the stock solution of KCl per liter, for comparison with sera on the basis of the assumption that all of the potassium in serum is ionized. This assumption is open to question.
‡ We have usually omitted magnesium from the solutions of reference.

The solutions of reference are quickly made up each day by pipetting the saturated basic solution into a series of flasks and adding the appropriate amounts of an accurately standardized solution containing approximately 102.7 mm per liter of CaCl₂. For observations on normal serum or cerebrospinal fluid a range of 0.8 to 1.5 mm per liter of calcium, at intervals of 0.1 mm per liter, will usually be adequate. The slight change of volume incidental to adding the amounts of CaCl₂ solution required is not sufficient to introduce a significant error.
**Procedure**

The heart is usually started with the solution of reference containing 1.2 mM per liter of calcium. After adjustments are made and the record started, the cannula is emptied and refilled with the same solution, in order to determine whether the heart has reached a stable condition. If a change in amplitude occurs, the cannula is emptied and refilled with the same solution, about once a minute, until the amplitude is stabilized.

The range of sensitivity is tested by changing the solutions. The change from one solution to another is effected by emptying the cannula with a medicine dropper, washing twice with the new solution, and filling with the new solution to exactly the same level as before, this level being most conveniently that at which the fluid just reaches the top of the cannula during contraction of the ventricle. The speed of reaction of the heart to a change in Ca++ of not more than 0.2 mM per liter is such that equilibrium with the new solution is frequently reached by the time the change of solution is completed.

For each preparation there is a certain minimum concentration of Ca++ which will provoke a maximum contraction. The method, therefore, is limited in its usefulness to solutions of fluids which provoke a response less than the maximum. Under favorable conditions the maximum response is not reached until the concentration of Ca++ is 1.5 mM per liter or higher, but individual preparations vary considerably. Solutions containing less than 0.5 mM per liter of Ca++ are not favorable to sustained action of the heart, and are unsuitable for general use. The most practicable range for observations by this method is 0.5 to 1.3 mM per liter of Ca++.

After the range of sensitivity is established, the fluid is again changed to one with a Ca++ concentration expected to be near that of the unknown. In the case of normal serum or cerebrospinal fluid this is about 1.2 mM per liter of Ca++. Having established equilibrium with this solution, a change is made to the unknown. The process is then continued, the known solutions being varied until the unknown is matched, and until the sensitivity is again established by demonstrating that the next higher solution will cause an increase and the next lower solution a decrease in amplitude. The concentration of calcium in the known solution
which matches the unknown may then be taken as the concentration of Ca++ in the unknown. If the unknown falls between two known solutions the concentration of Ca++ may be estimated to within 0.05 mM per liter. It is desirable to repeat each observation at least twice and preferably three times, in order to eliminate the influence of momentary fluctuations in the response of the heart.

In making the comparisons particular attention is paid to the height of systolic contraction, since the effect of varying Ca++ concentrations is more constant upon contraction than upon relaxation. Behavior during relaxation, however, serves as a useful check upon the condition of the heart and upon the uniformity of the conditions under which it is working.

When material is adequate, any solution which has been in the heart and cannula should be discarded. In the case of limited amounts of biological material the same fluid may be used repeatedly, provided that proper precautions are taken to avoid dilution in washing out the cannula. This is done by removing all that is possible of the previous solution before introducing the unknown, a procedure which is simplified by the fact that the heart empties itself almost completely when the cannula is empty. In this way observations may be made upon as little as 3 cc. of fluid. Observations have been made on a wide variety of solutions, containing various chemical substances, with little or no difficulty. With such solutions a favorable range of Ca++ concentrations can be chosen, and if any toxic effects exist, making the solutions unfit for use, they manifest themselves at once. The method is most accurate and offers the least difficulty with solutions of which the composition can be accurately controlled.

In the case of certain biological fluids difficulties are encountered which may result in loss in accuracy and in the ease with which observations are made. The chief sources of difficulty are as follows: (1) certain fluids, notably dog serum, are toxic, and the heart will survive only limited exposure to them; (2) the concentration of Ca++ in normal or hypercalcemic fluids may be at or above the upper limit of the range of sensitivity of the heart; (3) if hemolysis has occurred, the concentration of potassium in the serum may be too high; (4) most biological fluids contain substances which exert a pressor effect independently of the Ca++ con-
centration, and this effect must be differentiated from the Ca\(^{++}\) effect.

As a rule these difficulties can be overcome, but in rare instances no reading can be obtained. Cerebrospinal fluid from man and from dogs has caused relatively little difficulty. As to serum, the least difficulty has been encountered with human material. Rabbit serum is non-toxic, but on account of its high Ca\(^{++}\) content is apt to give rise to difficulties with the range of sensitivity. Dog serum is almost uniformly toxic, and the heart will survive exposure to it for only a few minutes. Beef serum has been found to be relatively unfavorable. Pleural fluid, chest fluid, and edema fluid of human origin have caused no difficulties. Serum from patients in uremia has not proved toxic, but there is doubt as to whether the standard solutions of reference are comparable to these sera. Aqueous humor, promptly removed from fresh slaughter-house material, is favorable. Chicken serum is satisfactory. All biological material should be used fresh and there should not be more than a faint tinge of hemolysis. Serum should not stand in contact with cells. Fluctuations in Ca\(^{++}\) concentration due to a moderate change in pH are not great, even in protein-containing fluids, but it is desirable to saturate the material with the CO\(_2\)-O\(_2\) mixture used for the standard solutions.

Granted non-toxic material, the most serious difficulty is with respect to the range of sensitivity, and a wide variation in behavior is seen in different preparations. Some preparations show a satisfactory range when fresh, others after a few minutes to an hour or so, others only after a night in the ice box. Still others show a satisfactory range in the standard solutions, but become insensitive in the higher ranges after exposure to the pressor effects of serum. Seasonal variations and the general condition of the frogs, plus individual variations, seem to play the greatest part in determining the range of sensitivity. The following suggestions may prove useful in case this difficulty is encountered: (1) select healthy frogs; (2) have patience in the hope that the range will improve; (3) expose the heart for brief periods to higher concentrations (2.0 mM per liter) of calcium; (4) discard the preparation in favor of a new one if it proves unsuitable. The sensitivity in the upper ranges may also be improved by lowering the temperature at which observations are made (see discussion of tem-
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Temperature). In general, we have found it possible to secure a range of sensitivity satisfactory with normal serum except for a period of a month or two just before freshly caught frogs are available in the spring. During this period the greatest difficulties with the range of sensitivity are encountered.

The difficulty with pressor effects, uncomplicated by difficulty with the range of sensitivity, is much more easily overcome. The effects of pressor substances so far observed differ from the effect of increasing Ca++ mainly in the speed with which the effect is produced and in the time necessary for it to disappear. The effect of a change in Ca++ concentration is very rapid in both directions, the new equilibrium being frequently reached, as above observed, by the time the change of solutions is completed. On the other hand, the maximum effect of a pressor substance may be obtained only after 2 or 3 minutes, and wears off very gradually when the pressor substance is removed. When in the course of an observation the effect of a pressor substance is seen, it is necessary only to wait until it reaches its maximum and then to proceed with comparisons at the new level. That this procedure is valid has been shown by experiments in which adrenalin has been added to artificially prepared solutions.

Subject to these limitations, reasonably satisfactory readings may be obtained with serum, and more satisfactory observations with cerebrospinal fluid and other protein-free or protein-poor fluids. The validity of the method in the case of serum has necessarily been based largely upon the collateral evidence obtained in investigations the results of which are to be published in detail shortly.

Sensitivity

Within its particular range every heart examined has been sensitive to changes in Ca++ concentrations. In the lower ranges a change of 0.1 mm per liter in Ca++ concentration may result in a change of 10 mm. or more in the height of the record, but as the upper limit of the range of sensitivity is approached, the same change in Ca++ concentration may produce a change of not more than 2 to 3 mm. in the record. Under favorable conditions and with Ca++ concentrations at about 1.0 mm per liter, the sensitivity, and hence the theoretical accuracy of the method, is to within
±0.1 mM per liter of Ca++, this sensitivity increasing with lower concentrations, so that there is a sensitivity and theoretical accuracy of ±10 per cent over the available range. Under the most favorable conditions, involving the use of solutions of identical composition except for Ca++ concentration, a sensitivity to differences of Ca++ concentrations of 0.025 mM per liter, or 1 mg. per liter, has been demonstrated, as is shown in Table III.

**Table III**

*Estimations of Ca++ in Solutions with Composition Unknown to Observer*

<table>
<thead>
<tr>
<th>Ca++ (= total Ca) present</th>
<th>Ca++ estimated</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>mM per l.</td>
<td>mM per l.</td>
<td>mM per l.</td>
</tr>
<tr>
<td>0.925</td>
<td>0.900</td>
<td>-0.025</td>
</tr>
<tr>
<td>0.675</td>
<td>0.700</td>
<td>+0.025</td>
</tr>
<tr>
<td>0.825</td>
<td>0.825</td>
<td></td>
</tr>
<tr>
<td>0.625</td>
<td>0.600</td>
<td>-0.025</td>
</tr>
<tr>
<td>0.775</td>
<td>0.800</td>
<td>+0.025</td>
</tr>
<tr>
<td>0.560</td>
<td>0.575</td>
<td>+0.015</td>
</tr>
</tbody>
</table>

**Temperature**

From the standpoint of the preparation the most satisfactory range of temperature is from 15–25°. Our observations have been made, as a rule, at room temperature, ordinarily between 22–25°. As the temperature rises above 25° the upper limit of the range of sensitivity tends to fall, and at about 30–32° the heart usually stops beating. At 15°, although the rate is slowed, the sensitivity in the upper ranges is usually increased.

The conditions governing ionization of calcium in serum or solutions containing protein are complex, and Ca++ concentrations observed at 25° can at best be considered only an approximation of those at 38°. Observations on undiluted serum at 15° have indicated Ca++ concentrations of 0.1 to 0.15 mM per liter higher than at 25°. The question of a temperature correction which may more accurately represent the conditions at 38° must remain open for the present.

**Variations in Solutions of Reference**

The solutions of reference described above are, as stated, based upon the average inorganic composition of the biological fluids.
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with which they are compared. Their composition represents our best judgment at the present time, based upon careful studies of the effects of varying each constituent independently, and it would appear that the use of these standard solutions of reference does not, under ordinary conditions, introduce errors of more than 0.1 mM per liter in the estimation of Ca\(^{++}\) in biological fluids of approximately normal inorganic composition.

It must be noted, however, that the amplitude of contraction of the frog's heart may change in response to the variation, in its nutrient fluid, of inorganic constituents other than calcium, and the effects of such variations are accordingly briefly noted.

Varying NaHCO\(_3\) at Constant CO\(_2\) Pressure—Variations in NaHCO\(_3\) sufficient to vary the pH between 7.35 and 7.6 when all solutions are saturated with the same CO\(_2\)-O\(_2\) mixture produce no appreciable effect.

Varying CO\(_2\) Pressure—With artificially prepared solutions, loss of CO\(_2\) after saturation, sufficient to raise the pH to about 7.6, has no appreciable effect. It is therefore unnecessary to take elaborate precautions to avoid loss of CO\(_2\) from the solutions of reference.

With artificially prepared solutions and with cerebrospinal fluid, reduction in pH below 7.35 by saturation with CO\(_2\) is accompanied by reduction in amplitude of the contraction of the heart. Between pH 7.35 and 7.6 there is no appreciable change. Within the range of pH usually encountered, therefore, the reaction of the fluid to be examined may be regarded as relatively unimportant. The same is true in the case of serum and other protein-containing fluids.

Magnesium—Mg\(^{++}\) has an effect similar to that of Ca\(^{++}\), but much weaker. The effect of 1.0 mM per liter of Mg\(^{++}\) is generally less than that of 0.05 mM per liter of Ca\(^{++}\). We have usually omitted magnesium from the solutions of reference.

Phosphates—Phosphates, in amounts up to 2.0 mM per liter, are without appreciable effect upon amplitude of contraction. In solutions containing 1.0 mM per liter of calcium, and more than 1.0 mM per liter of phosphate, a precipitate of Ca\(_3\)(PO\(_4\))\(_2\) is apt to be formed. We have usually omitted phosphates from the solutions of reference.

Potassium—The action of potassium is in part antagonistic to
the action of Ca++. The effect upon amplitude of contraction is variable. In general it may be said that a change of 1.0 mM per liter in potassium concentration may produce a change in amplitude of contraction, varying from no change to a change less than that produced by a change of 0.1 mM per liter of Ca++, but in the opposite direction.

Anions—In conformity with studies involving other biological material, the common anions appear to be without significant specific ion effects. In the case of the standard solutions of reference, variation in the proportion of Cl− and HCO3− is without effect, and other monovalent anions such as NO3− may be introduced without any change in the amplitude of contraction.

Isotonicity, Ionic Strength, and Sodium—As has been shown by various workers, substitution of isotonic sugar solutions for part of the NaCl content of the nutrient fluid, with other conditions remaining the same, causes an increase in the height of contraction of the frog’s heart, or an effect similar to that produced by an increase in Ca++ concentration. The substitution of an isotonic sugar solution for NaCl, while maintaining isotonicity, reduces the ionic strength of the solution as well as its NaCl content, and the increased contraction of the heart may therefore be due either to removal of sodium or to reduction in ionic strength, or to both. Clark (9) has shown that the effect is not due to a specific effect of sugar.

If the ionic strength and NaCl content of a solution are diminished by dilution with distilled water, still another factor is introduced, that of anisotonicity. The effect of changing from a solution isotonic with 0.9 per cent NaCl to one isotonic with 0.65 per cent NaCl is similar to the effect described above; i.e., it appears to enhance the action of Ca++. The effect of anisotonicity, however, has as yet not been separated from the effects of varying sodium content and ionic strength.

For the purposes of estimating Ca++ concentrations from their physiological activity, it is essential that ionic strength and sodium content be approximately equal in the known and unknown solutions, and that the solutions be isotonic.

SUMMARY

A method for the estimation of calcium ion concentration, in which the isolated heart of the frog is used as a biological indicator,
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is described. The method is applicable to any solution or biological fluid capable of sustaining the action of the frog's heart, provided that the concentration of calcium ions is within the range of sensitivity of the heart, and in the absence of a satisfactory chemical method for direct determination of calcium ion concentrations has proved useful in a number of chemical, physiological, and clinical problems.

BIBLIOGRAPHY

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