CEYLON J. MED. SCI. Vol. 19, No. 1, June 1970

A Rapid Method for the Separation of Protein-Bound and Ionized Calcium in Human Serum and the Establishment of the Clinical Norm for Ceylon Subjects

by

S. SENTHESHANMUGANATHAN & S. SACHITHANANDAM
Department of Biochemistry

AND

D. J. B. PERERA
Department of Clinical Pathology,
Medical Research Institute, Colombo 8, Ceylon.

UNIVERSITY OF CEYLON, COLOMBO
12 MAY 1971
MEDICAL LIBRARY

(With six text figures)

INTRODUCTION

The calcium in the plasma water is distributed in three forms viz. ionized (Ca^{++}) protein bound (PB-Ca) and complexed. (Fanconi & Rose, 1958; Rose, 1957). The accepted methods of determining the ionized calcium (Ca^{++}) of plasma are the biological method (Mc Lean & Hastings, 1934), and the chemical method (Rose, 1957). The use of ion exchange resins is another procedure (Slade, 1956).

Gel filtration with cross linked dextrans has been used widely in recent times for the separation of protein components from salts and low molecular weight compounds (Porath & Flodin, 1959; Kisliuk, 1960; Flodin, 1961). Recently, Jacobson and Widstrom (1962) described a very simple method for the separation of protein bound iodine components from non-protein ones. The method of Jacobson and Widstrom (1962) when applied for PB-Ca and Ca^{++} separations did not yield satisfactory results.

In this paper, a method is described to separate these two fractions. Gel filtration with cross linked dextran is introduced as a very simple and rapid method for the separation of both the PB-Ca and Ca^{++}.

MATERIALS AND METHODS

Materials: The gel used in this investigation is a polysaccharide made by cross linking dextran, manufactured by Pharmacia Ltd., Uppsala, Sweden, under the trade name 'Sephadex'. The following types were used G-25 fine, G-50 fine, G-75 (40—120μ) and G-100. All reagents used were of analytical grade unless otherwise stated. Ethylene

diamine tetracetic acid (EDTA) was obtained from British Drug Houses Ltd., Poole, U.K. All solutions were made in glass double distilled water and the preliminary experiments were conducted with pooled serum which were received in the Department of Biochemistry for biochemical analysis. Crystalline bovine serum albumin was obtained from Armour Pharmaceutical Co. Ltd., Eastbourne, U.K.

Preparation of solutions and indicator for the estimation of Ca by the EDTA titration: Solutions of EDTA and calcium were prepared as described (Varley 1962) and standardized using Erichrome Black T as the indicator (Baron & Bell 1957).

Collection of samples of blood for the establishment of the clinical norm for Ceylon subjects: total, protein bound and ionized Calcium: All blood samples were taken from volunteers, mainly the medical laboratory technologists, working at the Medical Research Institute, in the fasting state at about 9.00 a.m. except that a cup of tea was allowed about two to three hours before venepuncture. The subjects were allowed to rest and the sample of blood drawn without the application of a tourniquet. This process was strictly followed in the initial stages, since it was thought that the distribution of Ca in the two fractions, was affected by the acid-base balance. During the course of the investigations we observed that there was no appreciable change in the distribution of Ca in the fractions even in the blood drawn with the application of a tourniquet. In some cases in order to ascertain whether the atmospheric carbon dioxide would alter the distribution of calcium in the two fractions, the samples of blood were collected under liquid paraffin. In the latter cases, the dilution and the separation of sera were carried out under liquid paraffin. The only stage at which the samples came into contact with the atmosphere was when they were applied on the column.

Methods:

Estimation of calcium. In order to follow the appearance of Ca in the effluents, a rapid but reliable method had to be devised; for this purpose the EDTA titration procedure (W.H.O. 1963) was adopted with certain modifications.

The indicator solution was added to a solution containing buffer (5.0 ml) and glass double distilled water (45 ml). The colour of the resulting solution depended on the amount of metallic ions present. If the solution remained blue, the test solution containing the Ca was added and the titration carried out and completed with the least delay; the end point was a change from red-violet-to blue. If the solution containing the water, buffer and indicator was violet to red, the solution was titrated against the EDTA solution back to the blue colour. Subsequently the test solution was added and the titration completed. This procedure ensured a greater accuracy in the titration values. Under these conditions a blank was found to be unnecessary.

Proteins:

Proteins were estimated by a colorimetric method (Wolfson, Cohn, Calvary & Ichiba, 1948). The intensity of the colour was read on a Klett-Summerson photoelectric colorimeter using filter No. 54.

Preparation of the column and separation technique.

A column of 10 mm bore — 100 mm in length having a capacity of 8.5 ml was used hroughout the investigation. The gel was suspended in glass distilled water and stirred tthoroughly and the suspension added to the column. After the gel settled, more suspension of the gel was added until a length of 100 mm was attained. A wad of cotton wool was then placed on the surface of the bed of the column to avoid disturbing the gel during the addition of the serum or water.

The gel column was flushed with about 25 ml water before use. The serum (2.0 ml) was applied on the column and eluted with water. In the preliminary experiments 2.0 ml effluents were collected and the calcium and protein contents estimated in all the fractions. Elution with buffer solutions of varied pH values did not produce any change in the separation, hence water was used for eluting purposes. After standardising the method, only three fractions were collected (4.0, 5.0 and 8.0 ml). After the collection of 17 ml, the column was flushed with about 10 ml water. The column was ready for use again after-washing with about 10 ml water. We have used the same column more than 50 times without any significant error in the results. The time taken for one separation and estimation did not exceed 35 min.

RESULTS

Effect of adding calcium to a solution containing bovine serum albumin.

A solution containing albumin and calcium was prepared in physiological saline and its Ca and protein contents determined. This solution (2.0 ml) was applied on the column (G. 75) and eluted with water. Ten fractions, each of 2.0 ml, were collected and the protein and Ca contents determined. The results are provided in Fig. 1.

Effect of diluting serum on the separation of PB-Ca and Ca^{++}.

When the above experiment was carried out with pooled serum the result obtained for PB-Ca was found to be about 60% of the total Ca; a finding completely in disagreement with that of Rose (1957) and Fanconi & Rose (1958). These workers obtained a value of 40%. The experiment was repeated several times, with different batches of pooled serum, but with no change in the findings. It was thought at this stage that this could have been due to the viscosity of the serum and therefore we studied the effect of dilution.

The pooled serum was diluted with physiological saline and the experiments repeated both with the undiluted and diluted sera as described. The results (Fig. 2) indicate that the distribution of calcium in the protein-bound and ionized altered appreciably with dilution. A dilution of the serum two and half and over gave a clear separation of the two fractions. When G-50 was used in place of G-75 the protein commenced to appear in the effluent from the fifth ml rather than at the fourth. It is seen (Fig. 3) that Sephadex G-50 also gave results analogous to that obtained with G-75, but since the time taken for the separation with G-50 was less than that with G-75, we preferred to use G-50 for all further experiments.

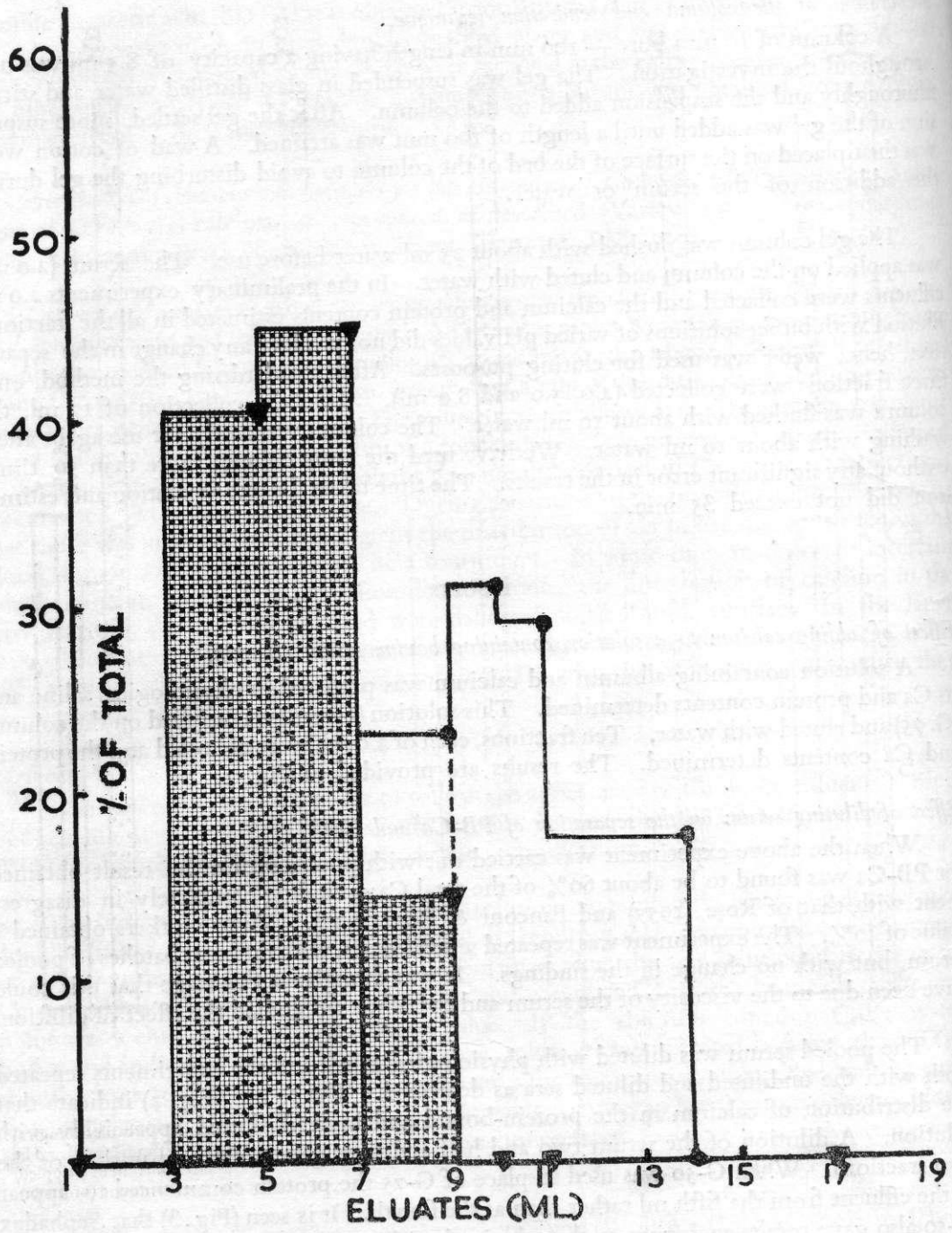

FIG. 1. Gel filtration in Sephadex-G. 75 column of 8.5 ml capacity. Calcium in the form of a solution was added to crystalline bovine serum albumin dissolved in physiological saline. The effluent was sampled in 2.0 ml fractions (absissa). The protein content (stippled) and the calcium (unstippled) were determined and expressed as per cent of the total amount (ordinate.)

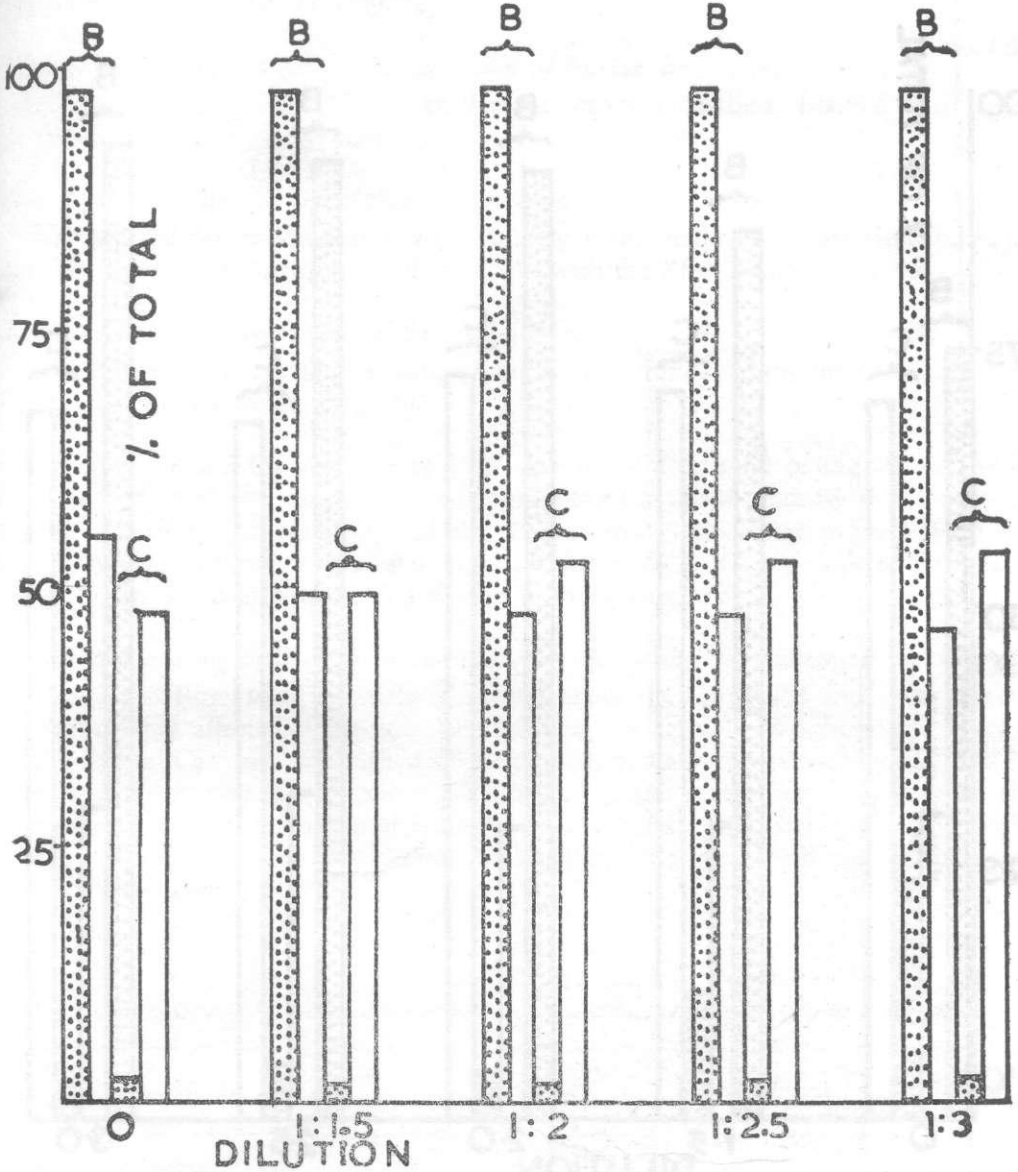

FIG. 2. Gel filtration of serum on sephadex-G. 75 column of 8.5 ml capacity. The serum was diluted with physiological saline; the dilutions are shown in the absissa. The effluent was sampled in three different fractions (3.0, 6.0 and 7.0 ml). The first fraction (3.0 ml) did not contain any protein or calcium hence not shown in the figure. The protein content (stippled) and the calcium (unstippled) were determined in the fractions and expressed as per cent of the total amount (ordinate). B—Second Fraction, C—Third fraction.

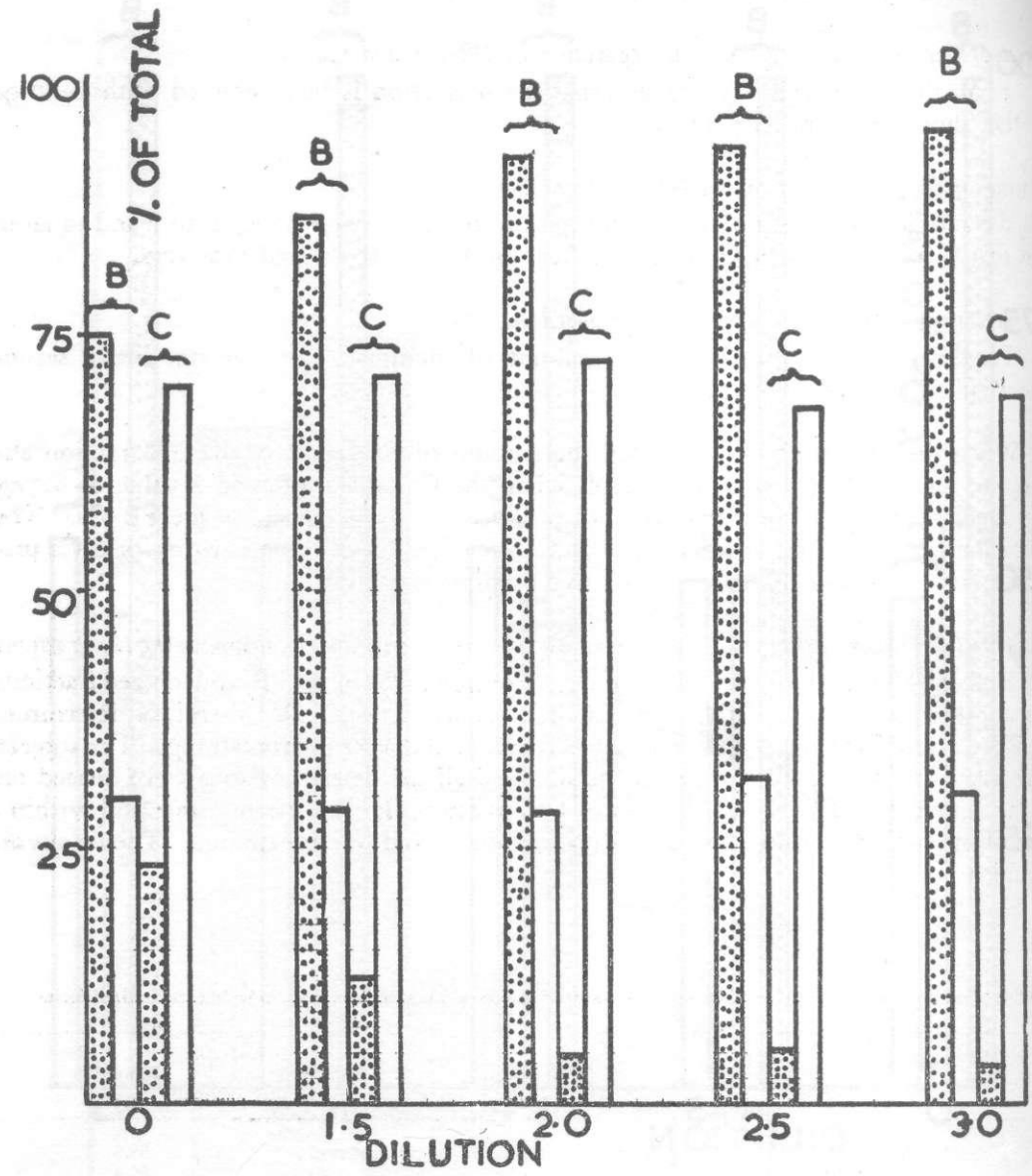

FIG. 3. Gel filtration on Sephadex-G. 50 column of 8.5 ml capacity of serum. The sample of serum was diluted with physiological saline; the dilutions are shown in the abscissa. The effluent was sampled in three different fractions (4.0, 5.0 and 8.0 ml). The first fraction (4.0 ml) did not contain any protein or calcium hence not shown in the diagram. The protein content (stippled) and the calcium (unstippled) were determined in the effluents and expressed as percentage of the total amount (ordinate). B—Second fraction and C—third fraction.

12 MAY 1971
MEDICAL LIBRARY

The separation of a sample of serum of a normal subject, under these conditions, with G-50 gel, is provided in (Fig. 4).

Effect of particle size of gel on the separation of PB-Ca and Ca^{++}.

From the results (Fig. 5) it is seen that the separation is best obtained with G-50 gel in the short time of 25 min.

Testing of the validity of the method of separation

We adopted two methods to test the validity of the method of separation and to ascertain that there was no admixture of the Ca^{++} with the PB-Ca and vice versa.

(*a*) *Recovery experiments with calcium added to serum*

For this study we added increasing amounts of calcium to a known amount of serum. The results are shown in (Fig. 6).

When the added Ca was increased the amount of Ca found in the PB-fraction also increased and reached a saturation level, when the Ca added attained a value of 625μg. Any increase of Ca above 625μg did not produce any more change in the PB-Ca. This saturation level, we infer as the total saturation level of Ca of the plasma to form Ca proteinate, as in the case of iron (Total iron binding capacity).

(*b*) *Effect of inducing alkalosis, by overbreathing, on the distribution of calcium in the two fractions.*

Fanconi & Rose (1958) found that the distribution of Ca in the PB and ionized fractions, was appreciably affected if the acid base balance was altered. We therefore determined the PB-Ca and Ca^{++} on two normal subjects before and after overbreathing. The subjects were asked to overbreathe for about 7-8 min and all the determinations were carried out on the same day. The samples of blood were taken under non fasting state and within a time of interval of 10 min between normal breathing and overbreathing. The results are provided in Table 1.

TABLE I

Effect of inducing alkalosis, by overbreathing, on the distribution of calcium in the protein bound and Ionized forms:

Cases		Total mg%	Calcium	
			Protein Bound (% of total)	Ionized
(1)	Normal	12.7	40.7	59.3
	Overbreathing	12.8	49.6	50.4
(2)	Normal	12.8	45.3	54.7
	Overbreathing	12.8	50.0	50.0

Samples of blood were drawn as described in the text. The diluted serum (2.0 ml) was applied on Sephadex G-50 fine gel column and eluted with water. The serum was diluted two and half fold with physiological saline. Collected three fractions from the effluent (4.0, 5.0 and 8.0 ml). The first fraction did not contain any calcium or protein; the mid fraction contained 96% of the total protein and a certain percentage of the total Ca; the third fraction contained the balance Ca and protein.

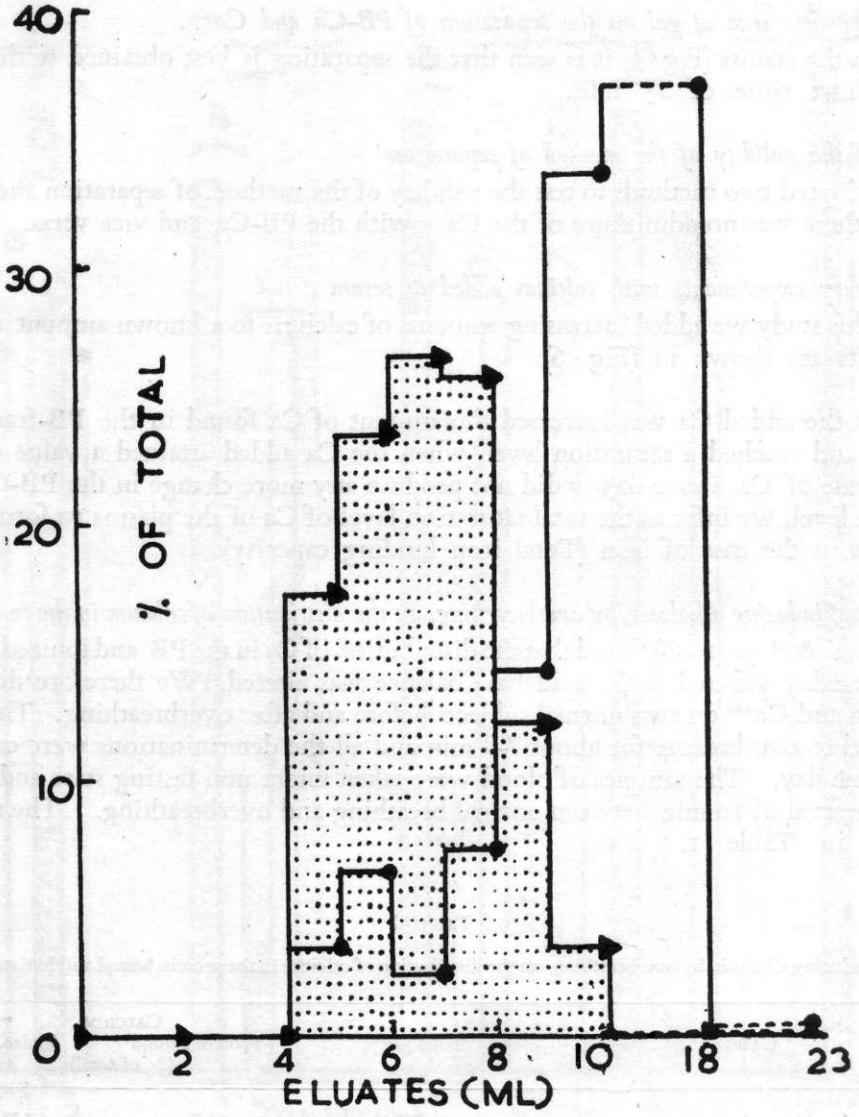

FIG. 4. Gel filtration of serum diluted two and a half fold with saline in 'Sephadex' G. 50 fine column of 8.5 ml volume. The effluent was sampled in 1 ml fraction (abscissa). The protein (stippled) and the calcium (unstippled) were determined in all the fractions and expressed as per cent of the total amount (ordinate).

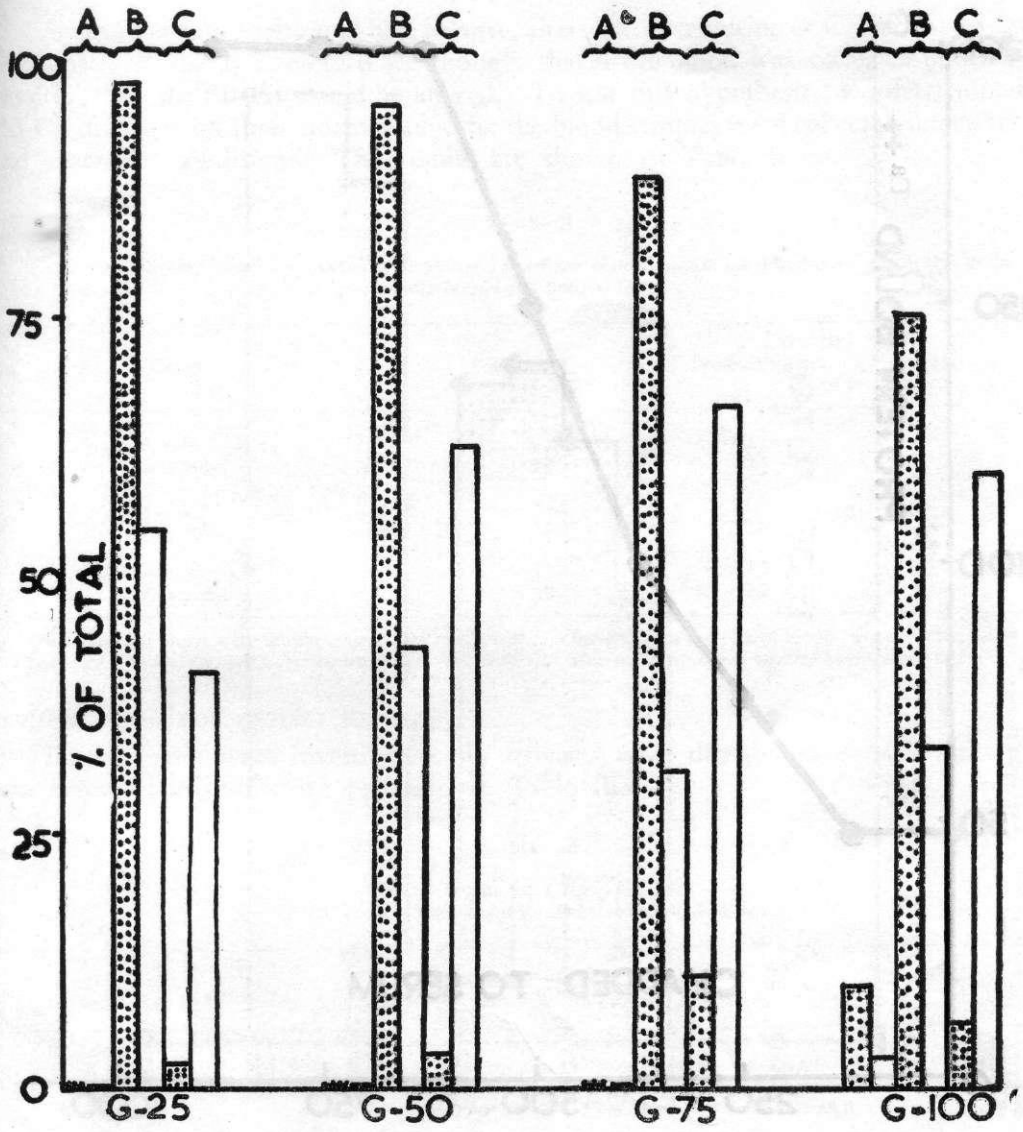

FIG. 5. *Effect of different particle size of Gel on the separation of protein bound calcium from the ionized form:* The gels used are shown in the diagram. The serum was diluted two and a half fold before the addition on the column. The effluent was sampled in three fractions (4.0, 5.0 and 8.0 ml). The protein content (stippled) and calcium (unstippled) were determined and expressed as percentage of the total amount (ordinate). A: First fraction (4.0 ml); B — Second fraction (5.0 ml); C — Third fraction (8.0 ml).

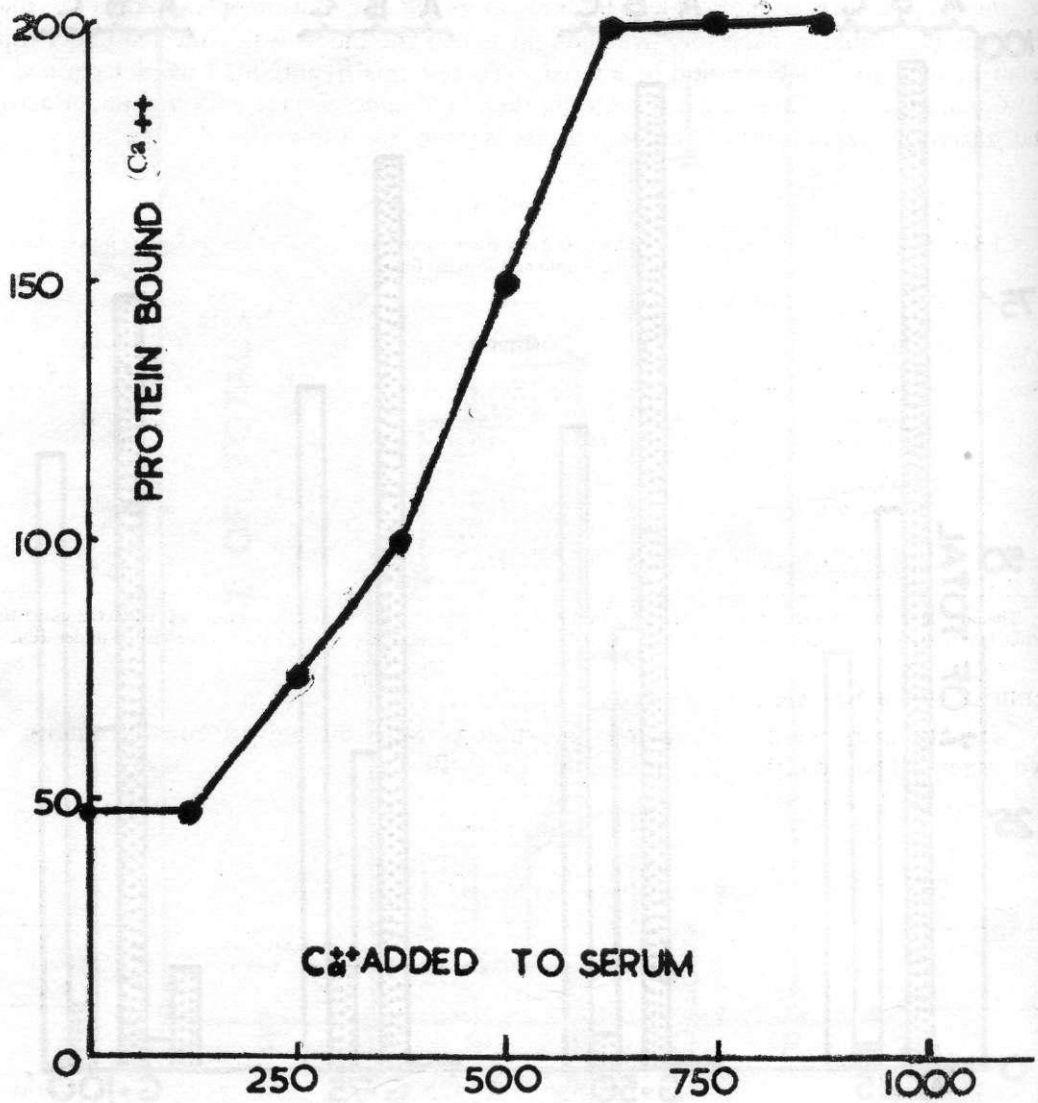

FIG. 6. Gel filtration of serum on sephadex G- 50 fine column of 8.5 ml of capacity. The calcium (abscissa) was added to the serum together with saline so that the final volume was 2.5 fold. The effluent was sampled in three fractions (4.0, 5.0 and 8.0 ml). The protein and calcium contents were determined in all the fractions. The protein bound calcium is expressed as the ordinate. The sample of blood used contained 48 μg calcium bo und to the protein.

Effect of collecting blood under aerobic and anaerobic condition on the distribution of Ca in the two fractions.

In vivo, a change in the acid base balance, alters the distribution of Ca in the two major fractions (2, Table I) Therefore we thought that if the blood was collected under liquid paraffin, then the PB-Ca would be altered. To test this hypothesis, we determined the PB-Ca and Ca^{++} on three normal subjects; the blood samples were collected under aerobic and anaerobic conditions. The results are shown in Table II.

TABLE II

Effect of collecting blood samples under aerobic and anaerobic conditions, on the distribution of calcium in the protein bound and Ionized forms :

CASES	Total mg%	CALCIUM Protein Bound (% of total Ca)	Ionized (% of total Ca)
1. Aerobic	12.9	40.7	59.3
Anaerobic	12.9	40.1	59.9
2. Aerobic	11.2	31.7	68.3
Anaerobic	11.2	32.0	68.0
3. Aerobic	12.4	37.9	62.1
Anaerobic	12.4	37.2	62.8

The samples of blood were drawn as described in the text. One set of the samples of blood was collected under liquid paraffin (anaerobic) and another set in the usual manner (aerobic). The separation was effected as described under Table I.

CLINICAL NORM FOR CEYLON SUBJECTS

Twenty cases were investigated, the subjects were distributed equally among the two sexes. The results are provided in Table III.

TABLE III

Clinical Norm for Ceylon Subjects.
Total, Protein Bound and Ionized Calcium and Protein.

	Total	CALCIUM Protein — Bound (% of the total)	Ionized	Protein g%
Males				
Mean	12.26	39.43	60.57	7.57
Standard Deviation	±0.56	±3.94	±3.94	±0.62
Range	11.2—12.9	32.0—45.3	54.7—68.0	6.37—8.64
Females				
Mean	12.19	38.38	61.62	7.69
S. D.	±0.40	±2.34	±2.34	±0.39
Range	11.5—12.8	35.9—42.7	57.3—65.8	7.20—8.52
Males & Females				
Mean	12.22	38.91	61.09	7.60
S. D.	±0.60	±3.34	±3.19	±0.52
Range	11.2—12.9	32.0—45.3	54.7—68.0	6.37—8.64

The samples were collected as described in the text. The dilution of the sera and the separation were effected as given in Table I.

DISCUSSION

The results of the present investigation indicate that the two major fractions, in which the calcium of the plasma water is distributed, are separable without admixture using either Sephadex G-50 fine or G-75 (40-120μ). The methods which are available at present are the biological and ultrafiltration (Mc Lean et al, 1934; Rose, 1957). Both these techniques are time consuming and/or expensive. Rose (1957) and Fanconi & Rose (1958) using the ultrafiltration technique obtained three fractions in which the Ca of the plasma water is distributed viz protein-bound, "complexed" and ionized. The complexed refers to that fraction of calcium which is ultrafiltrable but non ionized. By the present method of gel filtration we were able to obtain only two fractions and we are of the opinion that the complexed form appears along with the ionized fraction and that such a differentiation is not all that necessary since the clinical significance of differentiating the complex fraction, for diagnostic purposes, still remains uncertain.

In most of the blood samples separated and analysed for Ca and protein, we obtained about 2-6% of the total protein being carried with the ionized fraction. This, we are of the view, is not protein but some low molecular weight peptides; this is also estimated by the method used for protein determination viz colorimetry using an alkaline solution of $CuSO_4$; this reagent reacts with any compound which contains a peptide linkage-CO-NH-C. This aspect of the problem was not investigated further.

The validity of the method of separation was checked by one of two procedures. First by adding Ca to serum and following separation and secondly by inducing alkalosis by overbreathing. By both procedures we have shown without any reasonable doubt the validity of the method of separation. It should be pointed out at this stage that we were able to carry out the separations as well as the determination on the same day within a time interval of 3 hours. But Fanconi & Rose (1958) for technical reasons and in view of the long procedure of the ultrafiltration method could not carry out more than one determination of ionized Ca in any one day. The time interval was generally one week but was one day in one case and as long as three weeks in another case. The present results are therefore comparable with those of Fanconi & Rose (1958).

The results (Table I) obtained are in good agreement with those of the other workers (Fanconi &Rose 1958) in that the induction of alkalosis produced a shift of the ionized Ca towards the protein bound; thus indicating the validity of the method of separation as well as the method of estimation of calcium.

When increasing amounts of calcium is added to serum and the separation effected the amount of PB-Ca increased and reached a saturation level. The only explanation we could put forward for such a behaviour is that the additional Ca which appeared with the protein was not in an ionized form but in combination with the protein as calcium proteinate.

This phenomenon (saturation level) seems to be analogous to that of serum protein binding to iron giving rise to a saturation level which is referred to as Total Iron Binding

Capacity (TIBC). The clinical significance of Total Calcium Binding Capacity (TCBC) may be of some importance but this aspect of the problem was not pursued further.

It has been mentioned (Price, 1966) that in drawing blood for Ca estimation, extra care should be taken to avoid exercising and inducing alkalosis by the application of tourniquet. We therefore drew blood by venepuncture, from the same subject with and without the application of tourniquet and exercising the forearm. The samples of blood were drawn within an interval of 2 min and the separation as well as the estimation of Ca completed within 4 hours. The results obtained indicated that there was no significant change in the total, PB-Ca or ionized Ca under both condition.

In vitro the carbon dioxide present in the atmosphere has no influence in the distribution of Ca in the two fractions and hence there is no need to collect blood under liquid paraffin.

The clinical norm for Ceylon subjects was established by using twenty subjects, these were distributed equally among both the sexes. The total calcium ranges from 11.2 to 12.9 mg %. Our values are slightly higher than the values (Rose, 1957; Fanconi & Rose 1958) for British subjects. This we attribute to the method of estimation of total calcium. These authors used the precipitation method in which the calcium was precipitated as the oxalate and dissolved in dilute sulphuric acid and titrated against potassium permanganate solution. There are several drawbacks in such a procedure, firstly the precipitation might have been incomplete and secondly there was a lways a likelihood of loss of the calcium oxalate precipitate during washing. These may be the causes for their low values. These losses are overcome by the EDTA method. Further by carrying out the titration at the highly alkaline pH (12.66) we have eliminated any errors which would have been caused due to the presence of Mg ions. The presence of iron, copper and other metals have no influence on the calcium determination (WHO, 1963) under these conditions.

The values obtained for PB-Ca ranged from 32.0% to 45.3% while ionized calcium from 54.7% to 68 .0% of the total. Rose (1957) and Fanconi & Rose (1958) have reported the following figures for protien bound or non diffusible Ca from 35.0% to 40.0%and ionized calcium from 60.0% to 65.0%; these values are comparable with our results for ionized and protein bound Ca. We are of the opinion that the complexed form comes along with the ionized fraction and this would hence explain why we have been obtaining a small but appreciable amount of protien type of compounds (2-6% of total) in the ionized fraction.

We determined the mean as well as the standard deviation for the total, PB and ionized calcium for the sexes taken individually and together. The values are given in Table III. The 't' test was then applied and we obtained the following for total, PB and ionized calcium 0.3225, 0.7246 and 0.7246 respectively. There was no significant difference between the two groups.

The standard deviations obtained for total, PB and ionized calcium were very low, $\pm$ 0.60, $\pm$ 3.34 and $\pm$ 3.19 respectively, this therefore justifies our taking a small group of 20 to establish the clinical norm.

The results obtained in the present investigation indicate that the column efficiency increased with increasing particle size of the Sephadex gel and then decreased. Sephadex G-50 and G-75 gave the best separation without admixture of the two forms of Ca of plasma water. The procedure is limited by the viscosity of the sample. The latter finding agrees very well with those of Flodin (1961). Gel filtration of a protein solution is therefore equivalent to an exhaustive dialysis and is made in a short time. The gel could be used over and over again without any change in the performance of the column.

SUMMARY

A method is described by which the fractionation of protein bound and ionized calcium in serum can be performed with the same reliability as ultrafiltration, using gel filtration. The column efficiency depends largely in the particle size of the gel and the separation is limited by the viscosity of the solution. Sephadex G-50 and G-75 gave a good separation of the two forms.

In vivo, change in the acid-base balance produced a shift of the calcium from the ionized to the protein bound fraction but *in vitro*, the atmospheric CO_2 had no effect. The clinical norms for total, protein bound and ionized calcium for Ceylon subjects are reported.

ACKNOWLEDGEMENTS

The authors are indebted to those medical laboratory technologists of our Institute for voluntary blood donations; to Dr. V. Bardos, W.H.O. Consultant Virologist for the supply of Sephadex G-50 and G-75 to Mr. D. D. Jayatilleke for carrying out the statistical analysis and to Mr. P. S. B. Pathirana for the preparation of the figures.

REFERENCES

BARON, D. N., AND BELL, J. L. (1957). A Simple Specific Titration Method for Serum Calcium. *Clin. Chim. Acta.* **2**, 327—331.

FANCONI, A., AND ROSE, G. A. (1958). The ionized, complexed and protein bound fractions of Calcium in plasma. *Quart. J. Med.* **27**, 463—494.

FLODIN, P. (1961). Methodological aspects on gel filtration with special reference to desalting operations. *J. Chromatog.* **5**, 103—115.

JACOBSON, L., AND WIDSTROM, G. (1962). Separation of Iodine Compounds in serum by gel filtration *Scandiv. J. Clin. & Lab. Investigation* **14**, 285—288.

KISLIUK, R. L., (1960). A mild procedure for separating enzymes and Cofactors; *Biochem. Biophys. Acta.* **40**, 531—532.

MC LEAN, F. C., AND HASTINGS, A. B. (1934). A biological method for the Estimation of Calcium ion concentration. *J. biol. Chem.* **107**, 337—350.

PORATH, J. AND FLODIN, P. (1959). Gel filtration; A method for desalting and group separation. *Nature*, **183**, 1657—1659

PRICE, F. W., (1966). *A Text Book of the Practice of Medicine*, 10th ed. London. Oxford University Press.

ROSE, G. A., (1957). Determination of the ionized and ultrafiltrate calcium of normal human plasma. *Clin. Chem. Acta.* 2, 227—236.

SLADE, G. I. (1956). Application of a simple ion exchange resin technique to determination of serum or plasma protein-bound I—127 or protein bound I^{131}. *J. Clin. Endecr. Metab.*, **16**, 1122—1125.

VARLEY, H., (1962). *Practical Clinical Biochemistry*. 3rd. ed. p. 364, London, WILLIAM HEINEMANN, Medical Books Ltd.

World Health Organisation, Geneva (1963). *International standards for drinking water*. 2nd. ed. p. 122, World Health Organisation, Geneva, Switzerland.

WOLFSON, W. Q., COHN, C., CALVARY, E. & ICHIBA, F. (1948). A rapid method for the estimation of albumin, Total Globulin, Alpha-Globulin, Beta Globulin and Gamma Globulin in 1.0 ml Serum *Am. J. Clin. Path. Tech. Sect.*, **18**, 723—730.