The Relative Binding of Calcium and Strontium with Serum Proteins and Other Substances.* (27430)

CHARLES W. CARR, LEON SINGER, AND FRANCIS A. SPURRELL
Department of Physiological Chemistry, University of Minnesota Medical School, Minneapolis, and Dept. of Veterinary Surgery and Radiology, School of Veterinary Medicine, University of Minnesota, St. Paul

The relative binding of calcium and strontium as revealed by ultrafiltration is 1.2 in human serum(1) and 1.0 in rat serum(2). Whether the binding of calcium and strontium in serum of other species shows variation has not been determined, nor have the possible reasons for biological discrimination in the ultrafiltration of these ions from serum been explored. Since the binding of strontium and calcium with serum albumin at pH 7.4 is the same(3), speculation as to possible factors involved in the discrimination shown in human serum seems most likely related to other serum proteins. If other serum proteins can exert such an influence, it follows that differences in relative and absolute binding of the two ions in serum of various species would exist.

In an attempt to learn more about the relative and absolute binding of calcium and strontium in serum, experiments have been carried out on the ultrafilterability of these ions from whole sera and serum protein fractions of various species. A few other types of proteins and natural and synthetic polyelectrolytes were also tested.

Method. One volume of a mixture of Sr^{85} and Ca^{45} was added to 100 volumes of the protein solution to be studied. Ultrafiltrates of the protein solutions were obtained by a slight modification of a centrifugation procedure originally described by Clegg(4). Collodion membranes, test tube shaped (10 × 100 mm), and swelled in 97% ethanol(5) were used instead of cellophane because small volumes could be more conveniently handled. Ten ml aliquots of the solutions were placed in the membranes which were then centrifuged at slow speed (1500 rpm) for one hour; this resulted in formation of about 1-1.5 ml of ultrafiltrate.

One milliliter aliquots of solution were pipetted into stainless steel cups (1 × 5/16″), and Ca^{45} measurements were made with a thin mica window Geiger-Mueller counter. Appropriate correction for Sr^{85} in the samples was made by determining the number of counts obtained from the sample when unshielded and when covered with an aluminum shield 55 mg/cm^2 (about 0.025 mm) in thickness. This thickness of aluminum absorbed all beta counts and reduced the Sr^{85} counts about 50%. A standard Sr^{85} solution was counted with and without shield to determine the exact factor to be used in calculating from the shielded counts of the samples those in the unshielded counts that were due to Sr^{85}. These Sr^{85} counts were then subtracted from the total activity observed in the unshielded counts to give the activity of the Ca^{45} alone.

Sr^{85} was determined in similar aliquots of solution with a crystal scintillation counter not sensitive to beta particles. Appropriate corrections were made for resolving time and background. Data for the protein-containing solutions were adjusted for their water content so that they would be on a comparable basis with the data of the ultrafiltrates.

Materials. 20-30 ml of blood was obtained from animals of various species and was allowed to clot. The resulting serum was then tested for its relative binding of Ca and Sr. The serum protein fractions, obtained from Pentex, Inc., were prepared by cold ethanol fractionation. The casein, gastric mucin, chondroitin sulfate, deoxyribonucleic acid, and polygalacturonic acid were obtained from Nutritional Biochemicals Corp., polyacrylic acid from K and K Laboratories and polystyrene sulfonic acid from the Monsanto Co. Preparations of purified collagen were obtained through the courtesy of Dr. George

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Martin, National Institute of Dental Research, Bethesda, Md.

The solutions, other than the sera, were adjusted to pH 8.5 by addition of NaOH; NaCl and $CaCl_2$ were added to bring the final concentrations for sodium and calcium to 100 mM and 2 mM, respectively.

Results. Table I shows results obtained with sera from 6 different mammalian species. In columns 3 and 4 are listed the percentages of ultrafilterable Ca^{45} and Sr^{85}. These data have been converted to the ratio, % Ca bound/% Sr bound, by assuming that the non-ultrafilterable isotope represented the % of the cation that is bound. The values are listed in column 5. In all instances the binding of Ca is slightly greater than the binding of Sr. The same ratio and absolute values for ultrafilterable Ca and Sr in human serum have been reported by Samachson and Lederer(1).

It is of interest that although the ratio of binding is the same, there is some variation among the species in the absolute values. The data obtained for dog and horse sera were just about the same as that of the human, but the cow, sheep, and rat sera showed a somewhat higher degree of binding for both ions. Although the relative binding of the cations in human serum is similar to that found by Samachson and Lederer(1), the ratio obtained with pooled rat sera is higher than that reported by Likins, *et al.*(2).

Since it has been shown previously that Ca and Sr are bound to the same extent by serum albumin(3), further experiments have been carried out to determine which of the other major fractions of the serum proteins might be responsible for the slight but definite preference that is shown in whole serum for Ca-binding over Sr-binding. The results of these experiments with fractions of human and bovine serum are shown in Table II. It is seen that the equivalence of Ca and Sr binding with serum albumin (fraction V) is confirmed. With the other fractions, however, considerable variation occurs. For human serum, in all 3 fractions (IV, III & II) the ratio, Ca bound/Sr bound, is greater than 1.0, and thus could account for the ratio being greater than 1.0 in whole serum.

TABLE I. Relative Binding of Calcium and Strontium in Serum of Various Species.

Species	No. of sera analyzed	% ultrafilterable Ca^{45}	% ultrafilterable Sr^{85}	Ca bound / Sr bound
Human	2	42	55	1.29
Dog	1	44	51	1.14
Horse	3	43	52	1.19
Cow	7	32	41	1.15
Sheep	4	30	40	1.17
Rat	1*	32	40	1.13

* Pooled sera from 4 rats.

TABLE II. Relative Binding of Calcium and Strontium with Serum Protein Fractions, Human and Bovine.

Fraction	Species	% ultrafilterable Ca^{45}	% ultrafilterable Sr^{85}	Ca bound / Sr bound
V	Human	61	59	.95
	Bovine	57	58	1.02
IV	Human	47	58	1.26
	Bovine	82	68	.56
III	Human	53	63	1.27
	Bovine	50	43	.88
II	Human	61	67	1.18
	Bovine	52	59	1.17
Whole serum	Human	42	55	1.29
	Bovine	32	41	1.15

The results obtained with the bovine serum fractions are not quite so clear cut. For fractions IV and III the ratio, Ca bound/Sr bound, is actually reversed, *i.e.*, Sr is apparently bound to a greater extent than Ca. Only with fraction II is the ratio of the same order as it is in whole serum. This probably is due to the fact that paper electrophoretic analysis of our whole beef serum revealed that there is very little (*ca.* 10%) of the fractions IV and III.† Thus the ratio, Ca bound/Sr bound, for whole serum from the bovine is apparently accounted for by the relative binding to fraction II.

A further survey of fraction III for the pig and horse was also made. In both instances, the ratio, Ca bound/Sr bound, was 1.0 within the limits of error of the experiment. The

† This result for bovine serum is similar to that of Svensson(6) who reported that beef serum contains about 20% of the α- and β-globulins. However, Deutsch and Koenig(7) report that beef serum has 40% of these 2 fractions.

TABLE III. Relative Binding of Calcium and Strontium with Several Substances.

Substance	Conc., %	Ultrafilterable Ca^{45}	Ultrafilterable Sr^{85}	Ca bound / Sr bound
Serum albumin, Fract. V + phosphate, 2 mM	5.0	60	56	.91
Casein	5.0	11	18	1.08
Collagen, rat skin	2.0	98	97	—
Collagen, rat tail tendon	1.0	82	87	1.38
Gastric mucin	5.0	14	10	.96
Chondroitin sulfate	2.0	40	46	1.11
Deoxyribonucleic acid	1.0	17	15	.98
Polygalacturonic acid	.1	13	9	.96
Polyacrylic acid	.1	2	2	1.00
Polystyrene sulfonic acid	.1	27	25	.97

most striking feature, however, was that the ultrafilterable cation was 74% in the pig and only 5% in the horse. These results, when compared with those obtained with human and bovine fraction III (Table II), show that large variations can occur among different mammalian species with respect to this globulin fraction. On the basis of the results obtained with human and bovine fraction IV, it is anticipated that such wide variations among other species might also occur in this fraction. Thus, it would appear that the differences in Ca and Sr binding that occur in the sera of various species is due to the fluctuation in type and amount of protein occurring in fractions III and IV.

Another series of experiments, for comparative purposes, was carried out with a variety of proteins and other polyelectrolytes. These results are shown in Table III. In all instances the ratio, Ca bound/Sr bound, is unity within the experimental error although the % ultrafilterable cation varies from 100% for rat skin collagen to 2% for polyacrylic acid. Addition of 2 mM phosphate to bovine serum albumin did not change the ultrafilterable cation, and the phospho-protein, casein, did not show significant discrimination between Ca and Sr. The purified collagens exhibited little or no affinity for these cations; however, the mucoprotein, gastric mucin, had a high ability to bind them. The natural polyelectrolytes, chondroitin sulfate, DNA, and polygalacturonic acid bind Ca and Sr to a greater extent than most proteins, and the synthetic polyelectrolytes, polyacrylic acid and polystyrene sulfonic acid, also show a great affinity for these ions. The results obtained with gastric mucin and chondroitin sulfate give rise to the speculation that the large differences in the absolute amounts of cation binding by fractions III and IV may be due to fluctuations in amount of mucoproteins present. The over-all results, however, do not yield clues as to the possible types of proteins or types of groups in proteins that give rise to the slight but significant discrimination shown in fractions II, III, and IV. The reasons for the variations reported require further investigation.

Summary. Sera from various mammalian species, solutions of various fractions of serum proteins, and solutions of certain other substances have been tested by ultrafiltration in presence of Ca^{45} and Sr^{85} for their binding of Ca and Sr. For 6 species, human, dog, horse, cow, sheep, and rat, the ratio, Ca bound/Sr bound, was 1.29, 1.14, 1.19, 1.15, 1.17, and 1.13, respectively. The absolute degree of binding shown by these species falls into 2 groups. The human, dog, and horse sera bound the 2 ions to a lesser extent, 57% Ca and 47% Sr; cow, sheep, and rat sera bound the ions to a greater extent, 69% Ca and 60% Sr. Ultrafiltration of various serum protein fractions of the human and bovine revealed that fraction V (albumin) showed no difference between the binding of Ca and Sr but differences were noted with the globulin fractions (IV, III, II) that could account for the discrimination observed in whole serum. Results of the ultrafiltration of a variety of other proteins and polyelectrolytes led to the speculation that mucoproteins in the serum globulins could account for the large fluctuations in binding of both ions by globulin fractions of various species; however, the results did not show any indication of the type of substance or chemical grouping which could account for the slight but persitent discrimination between Ca and Sr that has been ob-

served in whole serum by ourselves and others.

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A Standardized Antigen and Skin Test for Toxoplasmosis.* (27431)

NANCY MITCHELL WENDE, JOSEPH S. HOUSTON† AND ROBERT B. DIENST
Department of Medical Microbiology and Public Health, Medical College of Georgia, Augusta, Ga.

The poor correlation between the presently used skin test(1) for toxoplasmosis and the more reliable methylene blue dye test for serum antibodies has been reported(2,3). This discrepancy may arise from use of an essentially unstandardized skin test antigen(4). One of the technical difficulties in securing a standardized antigen has been the efficient separation of the toxoplasma from the cells of the peritoneal exudate of the infected mouse.

The procedure described here provides a method to secure a skin test antigen essentially free of exudate cells and standardized by count of the toxoplasma. The use of this standard antigen made possible an investigation of standard procedures for reading and evaluating the skin test.

Preparation of skin test antigen. Aseptic technic, sterile solutions and sterile equipment were employed at all times. All centrifugations were in No. 1 International Centrifuge, rotating radius 20 cm.

The peritoneal exudate from 25 adult Webster mice, which had been infected 3 to 4 days previously with RH strain *T. gondii*, was collected into a 50-ml centrifuge tube and diluted 1:5 with saline. After centrifugation of this supension at 2500 rpm for 30 minutes, the supernate was removed. The solids were resuspended in one ml saline by expelling the total contents several times through a 27-gauge needle attached to a one-ml syringe. A 30-gauge needle was then substituted on the syringe and the total contents expelled 10-15 times while exerting as great pressure as possible on the plunger of the syringe and holding the needle at right angles to, and at a distance of one to two cm from the bottom of a 20-30-ml vaccine bottle. Air bubbles were allowed to escape between expulsions as foaming appeared to interfere with the splitting process. Rubber gloves were worn and work proceeded under a protective hood at this stage.

Microscopic examination determined the complete disruption of the exudate cells. The total contents were diluted with 10 cc saline, transferred to a 15-ml siliconized centrifuge tube with a water tight seal, and placed in a 56°C water bath for 60 minutes. Subsequently the solids were evenly dispersed by withdrawing and expelling the total contents several times through a 27-gauge needle on a 20-ml syringe. The cellular debris was separated from the toxoplasma by centrifugation at 600 rpm for 60 minutes and the supernate discarded. This procedure was repeated, if indicated, for better separation.

The toxoplasma were next suspended in a sufficient volume of saline to permit reliable counting with a haemacytometer, and total number of organisms was determined. The suspension was placed in a 56°C water bath for 60 minutes, then centrifuged in a 50-ml

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† Medical Staff, Gracewood State School and Hospital, Gracewood, Ga.