Effect of Chelating Agents on Uptake of Ca^{45} and Sr^{85} by Defatted Bone *in vitro*.* (26647)

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It has been shown that the ratio of uptake of Ca^{45} to Sr^{85} by bone is lower from serum or serum ultrafiltrates than from synthetic buffered solutions(1) and it has been suggested that this lowering of the ratio is due to the presence in the serum of proteins and natural chelating agents, the latter being present in serum ultrafiltrates as well. As serum proteins bind calcium more strongly than strontium(2), it seemed possible that natural chelating agents present in serum might behave similarly. Moreover, as strong synthetic chelating agents have been proposed for removal of bone-seeking radioisotopes, it seemed of interest despite emphasis on their limitations(3) to investigate their effect on the exchange between Sr^{85} and Ca^{45} in solution and bone calcium.

The ratio of Ca^{45}/Sr^{85} uptake provides a much more reproducible index than either the Ca^{45} or Sr^{85} uptakes individually(1) and permits detection of effects which might otherwise be unnoticed. Results are presented here of a study on the effect of chelating agents and other compounds on relative bone uptake of Ca^{45} and Sr^{85} and on the use of chelating agents to remove these isotopes from bone which had taken them up by exchange. Compounds naturally present in serum, such as citrate, were studied as well as synthetic chelating agents of the polyamino polyacid type.

Materials and methods. Beef femur was used as a source of bone. The marrow was removed and the bone was crushed, ground to between 60 and 100 mesh, defatted and dried at 105°C. Approximately 150 g were thus obtained and as 100 mg portions were used in each experiment, sufficient ground bone of the same batch was available for many studies. Sr^{85} and Ca^{45} were obtained as the chloride, Sr^{85} without carrier, Ca^{45} with a specific activity of approximately 30 μc/mg.

Solutions containing Sr^{85} and Ca^{45} plus carrier were buffered with 0.04 N veronal and hydrochloric acid to give a final pH of approximately 7.3. Carriers used were $SrCl_2$ or $CaCl_2$ or a mixture of both, each carrier in a concentration of 2.5 mM/L. One hundred mg samples of ground bone were shaken gently at 37°C for 2 hours with 50 ml of solution in flasks attached to a mechanical stirrer. The effect of strong chelating agents like ethylenediaminetetraacetic acid was studied in concentrations sufficient to chelate half the alkaline earth present, *i.e.*, 1.25 mM/L with Ca or Sr carrier, 2.5 mM/L with the combined carrier. The effect of citrate was studied at concentrations from 5 to 25 mg%, of glucose at 100 mg% and of bicarbonate at 25 mM/L. The pH of the bicarbonate solutions was reduced to 7.2 or 7.3 before the experiment by passage of oxygen containing 5% CO_2, and was redetermined after shaking to insure that there had been no appreciable loss of CO_2. A combination of bicarbonate at the same concentration and phosphate at a concentration of 3 mg % was also studied.

At the end of 2 hours of shaking, the bone was ashed and analyzed for Ca, P, Sr^{85} and Ca^{45}. Sr^{85} and Ca^{45} determinations were also carried out on the original solution and on the solution after shaking with bone, while P was determined on the buffered solutions after shaking and Ca on solutions in which no Sr was present. Uptake of Sr^{85} and Ca^{45} was expressed as percent in bone compared to the amount present in the original solution. Analyses for Sr^{85} and Ca^{45} in solution were carried out to insure complete recovery of the radioisotopes and were not used to calculate uptakes, as difference between the original solution and the solution after 2 hours of shaking was much more subject to error than direct determination of the radio-

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isotope in bone. Analyses for Ca and P were likewise carried out to insure that there was complete recovery of the bone and no appreciable loss of bone into the solution.

Sr^{85} was determined in a scintillation counter of the well type, Ca^{45} by precipitation with stable Ca as previously reported (4), counting being done in a thin-window gas flow counter, stable calcium by the method of Shohl and Pedley(5), phosphorus by the method of Fiske and SubbaRow(6).

Experiments on removal of radioisotopes from bone were carried out by shaking 100 mg bone samples with Ca^{45} and Sr^{85} for 2 hours in 50 ml of buffered solutions containing no carrier and then shaking the bone samples for another 2 hours with fresh 50 ml samples of buffered solutions containing 2.5 mM/L $CaCl_2$ and a chelating agent. Only those chelating agents which had shown an effect in the previous part of the study were used in these experiments and, as before, the strong chelating agents were used in concentrations of 1.25 mM/L, citric acid in a concentration of 25 mg %. In addition to the usual analyses, the solution used for removal of the radioisotopes was also analyzed for Sr^{85} and Ca^{45}.

Studies were carried out at least in duplicate and in a few cases in triplicate or quadruplicate. To take account of the effects of variations in shaking rate and of other factors which might vary from day to day, duplicates were not usually done on the same day, except in those cases where the calcium or strontium salt of the chelate appeared to form a supersaturated solution. In such cases, duplicate experiments were performed at the same time, immediately after preparation of the chelate solution, before precipitation could take place.

In the experiments on radioisotope uptake, all compounds were studied with at least 2 of the 3 carriers (Ca, Sr, Ca + Sr). In most cases, when results with the chelating agents differed from those of the controls, the differences were obvious without statistical analysis. Nevertheless, to provide an objective test for doubtful cases, all Ca^{45}/Sr^{85} ratios obtained with each carrier were grouped together on the asumption that whether or not they were samples of different populations, they had the same standard deviation. The assumption was obviously valid for those compounds which had no effect on uptake and, as approximately half the compounds studied were of this nature, the result was to provide many replicates of a control with a good estimate of its standard deviation. The formula used was

$$\sigma^2 = \frac{1}{N-k} \sum_{j=1}^{k} \sum_{i=1}^{n_j} (x_{ij} - \bar{x}_j)^2 \qquad (7)$$

where σ is the standard deviation, x_{ij} is observation i in sample j, $\bar{x}_j$ is the mean of sample j, with n_j observations, k is the number of samples, and N total number of observations. In the present study, k is the number of compounds studied (including control), each value of j refers to a particular compound, and n_j varied from 2 to 4.

Results were considered different from the control at the 0.05 level of significance if they differed by at least 2 standard deviations. As 123 experiments were carried out, a small number of findings of significance might be expected where no significance was actually present. The possibility of such errors was reduced by considering results for a given chelating agent to be positive only when significant differences were found with all the carriers used.

Results. Table I shows the effect of strong chelating agents in reducing the ratio of Ca^{45}/Sr^{85} uptake by bone from solutions containing Ca carrier. Although the amount of uptake, as previously noted(1), was subject to considerable variation, reduction of the Ca^{45}/Sr^{85} ratio from 1.28 to 1.01 or less was a reliable indication of the effect of the chelating agents at a level of significance far beyond the 0.05 value suggested in this table. No effects were shown by glucose, lactate, gluconate, bicarbonate and the bicarbonate-phosphate mixture.

Table II shows similar effects for the same chelating agents as well as for isopropylenediaminetetraacetic acid (IPDTA) when the radioactive solution contained Ca plus Sr carrier or Sr carrier alone. The effect was even more pronounced in the presence of these carriers than in the case of Ca carrier,

TABLE I. Uptake of Ca^{45} and Sr^{85} by Bone from Solutions Containing Strong Chelates and Ca Carrier.

Chelate	Uptake by bone		
	% Ca^{45}	% Sr^{85}	Ca^{45}/Sr^{85}
None	12 8	10.0	1.28
Bis (2-aminoethyl) ether tetraacetic acid (BAETA)	12.3	12.2	1.01
Cyclohexane trans 1,2-diaminetetraacetic acid (CDTA)	11.8	13.8	.86
Ethylenediaminetetraacetic acid (EDTA)	13.4	14.1	.95
Diethylenetriaminepentaacetic acid (DTPA)	10.7	10.9	.98

Stand. dev. for Ca^{45}/Sr^{85} ratios for Ca carrier, calculated in manner given in text, was 0.04. Values of 1.19 or less, significantly different from control at 0.05 level. No effect shown by glucose, lactate, gluconate, bicarbonate, bicarbonate plus phosphate.

TABLE II. Uptake of Sr^{85} and Ca^{45} by Bone from Solutions Containing Strong Chelates and (Ca plus Sr) Carrier or Sr Carrier.

Chelate	Ca plus Sr carrier			Sr carrier		
	% Ca^{45}	% Sr^{85}	Ca^{45}/Sr^{85}	Ca^{45}	Sr^{85}	Ca^{45}/Sr^{85}
None	11.9	6.5	1.83	21.7	10.1	2.12
BAETA	8.8	7.4	1.19	13.6	9.9	1.37
CDTAδ	7.7	8.8	.88	6.5	10.1	.65
EDTA	7.7	8.1	.95	7.4	9.7	.77
DTPA	7.6	6.9	1.11	9.8	9.2	1.07
IPDTA*	8.5	10.6	.80	10.5	14.1	.75

* Isopropylenediaminetetraacetic acid.

Stand. dev. for Ca^{45}/Sr^{85} ratios, Ca plus Sr carrier, was 0.11 and all values of 1.60 or less differ significantly at 0.05 level.

Stand. dev. for Ca^{45}/Sr^{85} ratios, Sr carrier, was 0.15 and all values of 1.81 or less differ significantly at 0.05 level. No effect shown by glucose, lactate, gluconate, glutamate, aspartate, borate, lysine, glutathione, glycerophosphate, bicarbonate, bicarbonate plus phosphate.

the ratio for Sr carrier being reduced from 2.12 for the control to as little as 0.65 for cyclohexanediaminetetraacetic acid (CDTA). In Table II, the effect of the chelating agents is evident not only on the Ca^{45}/Sr^{85} ratios but on the individual radioisotope uptakes as well, that of Sr^{85} being apparently unaffected, or in the case of IPDTA possibly increased slightly, while uptake of Ca^{45} was decreased. Again no effect was shown by weak chelating agents listed below Table I or by glutamate, aspartate, borate, lysine, glycerophosphate or glutathione.

Table III shows the effect of various concentrations of citric acid and adenosine triphosphate. In the presence of Ca carrier, 10 mg % citrate reduced the ratio significantly, while in the presence of the other carriers, 25 mg% citrate was needed to obtain a definite effect. Adenosine triphosphate was effective with all 3 carriers at both concentrations used.

Table IV gives stability constants for Ca and Sr. complexes of various compounds taken from the literature(8,9,10). Those compounds which were without effect on uptake ratio had stability constants of the order of 1 or 2, while citric acid and ATP, which had a definite but moderate effect, had constants between 3 and 4 and chelating agents with the greatest effect had stability

TABLE III. Ratios of Ca^{45} and Sr^{85} Uptake by Bone from Solutions Containing Citrate or Adenosine Triphosphate with Ca (Ca plus Sr) or Sr Carriers.

Chelate	Ca^{45}/Sr^{85} ratios		
	Ca carrier	Ca + Sr	Sr carrier
None	1.28	1.83	2.12
Citrate, 5 mg %	1.20	1.88	2.18
" 10 "	1.17	1.90	2.11
" 20 "	1.03	1.71	1.92
" 25 "	1.04	1.32	1.65
ATP, to chelate ¼ of carrier	1.14	1.47	1.78
ATP, to chelate ½ of carrier	.93	1.30	1.54

Differences significant at .05 level when values for Ca carrier 1.19 or less, Ca plus Sr carrier 1.60 or less, Sr carrier 1.81 or less.

TABLE IV. Stability Constants of Compounds Used to Modify Bone Uptake.

Compounds		pK (Ca)	pK (Sr)
ATP	(9)	3.60	—
Citric acid	(8,9)	3.22	2.7
Gluconic "	(8,9)	1.21	1.00
Lactic "	(8,9)	1.07	.70
Glutamic "	(8,9)	1.43	1.37
Aspartic "	(8)	1.60	1.48
Diethyl barbituric acid	(8)	.66	.48
BAETA	(8)	10.05	9.34
CDTA	(9)	12.50	—
EDTA	(8,9)	10.59	8.63
DTPA	(8,9)	10.11	9.68
IPDTA	(10)	10.4	10.7

Stability constants vary with ionic strength, temperature and other variables but above values permit comparison of Ca and Sr binding by same compound.

constants of the order of 10. The constants cited were greater for Ca than for Sr in all cases except that of IPDTA.

Table V shows the effect of chelating agents added to solutions in order to remove Ca^{45} and Sr^{85} from bone by exchange. In the absence of chelating agents, Ca^{45} was removed from bone less readily than Sr^{85}. Addition of chelating agents to the wash solutions increased the relative removal of Ca^{45} in all cases listed in the table except that of citric acid, which showed a doubtful effect. There appeared to be either unchanged or slightly increased removal of Ca^{45}, as shown in the first column of the table, accompanied by decreased removal of Sr^{85}.

Discussion. From the results obtained, it appears that the Ca^{45}/Sr^{85} uptake ratio found under the present experimental conditions may be used as a criterion of the difference in binding of Ca and Sr by a chelating agent. All of the strong chelating agents used in this study, except IPDTA, are reported to have greater stability constants for Ca than for Sr, and in view of the fact that IPDTA decreased relative Ca^{45} uptake at least as well as EDTA, CDTA and the other strong chelates, the stability data reported for this compound may well be erroneous. The uptake data for IPDTA are difficult to explain on the basis of a greater stability constant for Sr.

At the concentrations used, practically all the chelating agent was in combined form. In the presence of Ca or Ca plus Sr carrier, the calcium complex was chiefly formed. With EDTA, for example, whose Ca and Sr stability constants differ by 2 units, the amount of Ca chelate formed was approximately 100 times that of the Sr chelate. With other chelating agents, ratio of Ca chelate to Sr chelate was lower but still much greater than 1.

The preferential sequestration of Ca ions resulting from the greater stability constant for Ca resulted in a decrease in the uptake ratio of Ca^{45}/Sr^{85} but not in any obvious relation to the decrease of Ca ions. In the case of Ca carrier, where approximately half the calcium was bound and little of the Sr^{85}, the greatest decrease in ratio was shown by CDTA, which gave a value approximately two-thirds that of the control. In the case of Sr carrier, where all the Ca was bound and half the Sr^{85}, the ratio was reduced to about one-third that of the control.

The reason for a lack of parallelism in Ca^{45} exchange and Ca ion concentration must be sought in the nature of the exchange reaction. Neuman and Neuman(11) believe that with bone mineral, the first step is an exchange of ions between bulk solution and hydration shell of the bone crystal. Chelation obviously affects this exchange, but as chelated Ca and Sr may also penetrate the hydration shell, the net effect on this first step may be minor. The second step suggested(11) is an exchange between hydration shell and crystal surface itself and here the effect of chelate may be more pronounced. Ca ions in the

TABLE V. Ca^{45} and Sr^{85} Removed from Bone by Solutions Containing Stable Ca and Chelates.

Chelate	% Ca^{45} removed	% Sr^{85} removed	Ca^{45}/Sr^{85}
None	45.5	49.7	.92
EDTA	43.7	28.7	1.52
DTPA	52.4	40.9	1.28
BAETA	53.4	44.7	1.20
IPDTA	55.2	31.7	1.71
Citrate	36.8	38.6	.96
ATP	38.7	32.7	1.19

Ca^{45} values significantly different at .05 level outside of range 38.7 to 52.3.

Sr^{85} values significantly different at .05 level outside of range 45.7 to 53.7.

Ca^{45}/Sr^{85} ratios significantly different at .05 level above .96.

crystal surface may exchange directly with Ca ions in the hydration shell and therefore more rapidly than with chelated Ca.

Subsequent steps, which involve interchange between surface positions and crystal interior, along with possible remodeling and recrystallization, are of no importance in the short periods here under consideration. However, even the first 2 steps overlap and complications are introduced, as Dallemagne and his coworkers have emphasized(12,13,14), by the organic phase of bone. Although Ca^{45} and Sr^{85} do not concentrate in the organic phase, it is the medium in which ions diffuse (12). Ca and Sr ions are bound to some extent by this organic phase(1), so that diffusion through it is not merely a physical process but is accompanied by continual binding and release of alkaline earth ions. Chelating agents affect this process by lowering the concentration of free alkaline earth ions but may to some extent supplement it with the diffusion of Ca and Sr chelates.

In the overall picture, therefore, a net effect is evident even with such moderately strong chelating agents as citric acid and ATP. Apparently, however, compounds which form complexes with stability constants for Ca and Sr too close together do not affect the ratio. (It may be noted that the stability constants for diethylbarbituric acid are below 1 and that use of this compound as a buffer, therefore, does not affect the results.) As citric acid and ATP are both present in serum, although in smaller amounts than here used, and as other natural chelating agents may also be present, results support the assumption that natural chelating agents are at least partly responsible for the low Ca^{45}/Sr^{85} uptake ratio from serum and ultrafiltrate compared to uptake from synthetic buffered solutions.

It has previously been shown that the presence of phosphate does not affect the Ca^{45}/Sr^{85} ratio(1). The lack of effect of bicarbonate, as well as of bicarbonate-phosphate mixtures, is of interest, as calcium phosphate and calcium phosphate-bicarbonate complexes may exist in serum(15). If they do exist, their failure to affect the ratio may be due to the low order of the calcium stability constant, the closeness of values for Ca and Sr stability, or both factors.

The effect of strong chelating agents on the relative removal of Sr^{85} and Ca^{45} by exchange is important. It has been shown that because of the preferential chelation of Ca compared to Sr, the sodium salt of EDTA actually retards renal elimination of Sr^{85} (16). Although this unfavorable effect can to some extent be overcome by using the Ca salt instead of the Na salt, and substituting other chelating agents for EDTA, the preferential chelation of Ca still causes difficulties. It is now evident that strong chelates also decrease relative removal of Sr^{85} from bone by exchange. It must be emphasized that these data obtained *in vitro,* are not directly applicable to the living organism, where accretion of bone containing radioisotopes takes place, and dissolution of bone crystals is needed to remove the radioisotopes. However, as chelates appear to be potentially most effective shortly after administration of a dose, before much accretion of bone has taken place, the attempted removal of radiostrontium with chelating agents must still contend with the unfavorable effect of chelates on exchange, unless chelating agents with stability constants for Sr approaching or exceeding those for Ca are developed.

Summary. The presence of chelating agents in buffered solutions affected the relative uptake of Ca^{45} and Sr^{85} by defatted bone powder. Strong chelating agents, like ethylenediaminetetraacetic acid and cyclohexanediaminetetraacetic acid, decreased the ratio of Ca^{45}/Sr^{85} uptake considerably in presence of Ca, Ca plus Sr, or Sr carrier. Citrate and adenosinetriphosphate had similar but weaker effects. No effect was shown by glucose, lactate, gluconate, bicarbonate, bicarbonate plus phosphate, glutamate, aspartate, borate, glycerophosphate, lysine or glutathione. Those compounds which showed no effect had stability constants for Ca of less than 3. Strong chelating agents also decreased the relative amount of Sr^{85} removed from defatted bone powder by exchange. Results indicate that natural chelating agents may be partly responsible for the low Ca^{45}/Sr^{85} uptake ratio by bone from serum com-

pared with uptake from synthetic inorganic solutions and emphasize the difficulty of removing Sr^{85} from bone with chelating agents now available.

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Four Newly Recognized Adenoviruses. (26648)

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During a longitudinal study of the microbial experiences of children in an institution in Washington, D. C.(1) more than 50 strains of adenoviruses were isolated which could not be identified as any of the known types(2,3). Further study revealed that these viruses could be grouped into the 4 previously unrecognized serotypes which are described in this report.

Materials and methods. Prototype adenovirus strains were obtained from Drs. W. P. Rowe and S. D. Bell, Jr. The "S-1058" strain of type 7A was used as the representative of type 7. Neutralization tests were carried out in Rhesus kidney cell cultures(2) employing "procedure 2" of Rowe, *et al.*(4). Hemagglutination and hemagglutination-inhibition (HI) tests were performed as described previously(5). Rabbit antisera were used in both neutralization and HI tests(5). Complement-fixation tests were done by a standard technic which has been used with a number of viral antigens(4).

Throat and anal swabs were collected, stored, and inoculated by the methods outlined previously(6). Isolates were recovered in a variety of tissue cultures of human origin maintained for varying periods of time on several different kinds of media. At least one strain of each type, however, was isolated in either HeLa or KB cells. The latter tissues had been grown on a medium consisting of Eagle's basal medium with 10% inactivated human serum and were maintained on medium No. 199 with 5% inactivated chicken serum. After inoculation, KB and HeLa cultures were observed for cytopathic effects (CPE) for 7 to 12 days, when at least one blind passage was made. Maintenance fluid was usually changed every 2 or 3 days, but in some instances changes were omitted in cultures observed for only 7 days.

Results. The viruses described here appeared to be less readily isolated from a given specimen in this study than did adenovirus types 1, 2, 3, and 5. In general, the CPE of the new serotypes was slower to appear than that of the latter viruses and in