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Studies on Chloride of Bone in Cat and Rat.* (24208)

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The chloride space, calculated from values for bone chloride content, is frequently used as a measure of extracellular fluid volume of bone. Use of this calculation rests on the assumption that chloride is not located in cells or on crystals but solely in extracellular fluid. However, it has been observed by Nichols and Nichols(1) in dogs and by ourselves in rats, that the chloride space is often greater than one would expect if total bone water content was extracellular fluid. This discrepancy is complicated by the difficulties of chloride analysis in a tissue that contains small amounts of chloride with the possibility of associated errors due to blood content of cortical bone or contamination of bone samples with marrow and blood.

The purpose of this paper is to present the results, in cats and rats, of an accurate method of chloride analysis for cortical bone. Use was made of Br^{82} to determine exchangeability of the chloride and as a check on the accuracy of chloride analysis. Cr^{51} labeled red cells were also used to determine contributions made by blood content of bone to its water and chloride content.

Procedure. Cats of varying weights but unknown ages were anesthetized with Dial with Urethane (Ciba) injected intra-abdominally. Venesections were made in both saphenous veins. A solution of KBr^{82}‡ in normal saline and a suspension of Cr^{51} labeled red cells were injected into one saphenous vein by the method of Barnett and Fellers(2), using a maximum of 8 ml of normal saline as wash solution. The other venesection was used solely for withdrawal of samples. Previously an aliquot of the KBr^{82} solution had been removed for counting as a standard, leaving 10 μc to be injected. The Cr^{51} labeled cell suspension was prepared by withdrawing 3 ml of blood and incubating it for 30 minutes at room temperature with approximately 100 μc of Cr^{51} in the form of $Na_2Cr_2O_7$.§ Red cells were washed 3 times with normal saline, reconstituted to original volume, an aliquot removed for counting as a standard and the remaining cell suspension injected into the cat. Two hours were allowed for equilibration of both isotopes, since previously determined disappearance curves of both KBr^{82} and Cr^{51} labeled red cells, injected intravenously, reached a constant slope by 2 hours after injection. At the end of this period, blood was withdrawn for counting and analysis. At the same time the blood supply of left upper extremity was occluded with a tourniquet and the limb amputated at elbow. The radius and ulna were dissected free, the periosteum removed by scraping and the marrow blown out with compressed air. The bone was then broken into small pieces, cleaned of remaining marrow by blotting with filter paper, placed in a tared vial and weighed immediately. For determination of chloride content of rat bone, groups of Holtzman rats of known ages were exsanguinated after ether anesthesia. All long bones of young rats were removed but, in older animals, only the right upper and lower extremities were taken. Before weighing in tared bottles, the bones were cleaned using the same procedure as for cat bone.

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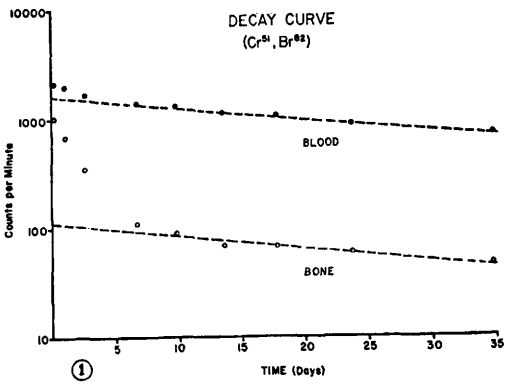
Analytical methods. Blood and plasma water were determined by drying. Plasma chloride was determined by Volhard titration as modified by Van Slyke(3). Bone water was determined by drying at 65°C *in vacuo* for 48 hours. Fat was extracted from cat bones with dry ethyl ether at room temperature but was not extracted from rat bone, since the fat content was less than 2% in preliminary extractions. The bones were then ground to a fine powder with a mortar and pestle, placed in platinum crucibles, redried and weighed. A modification of the method of Cheek and West(4) was used to determine bone chloride as follows: Approximately 0.5 ml of 2N KOH (Special pellets—Low in Chloride, Baker)/150 mg of dry bone was added to the crucible contents. The crucibles were placed on steam bath for at least one hour until dry and then in a muffle furnace at 550°C overnight. Upon cooling, $AgNO_3$-HNO_3 was added and the solutions titrated in duplicate with KCNS using Ferric Alum Indicator. These solutions were colorless and the end points distinct. A blank determination on the KOH revealed no detectable chloride. Ash content was determined by ashing another aliquot of bone powder at 525°C. Determination of radio-activity in the samples was made with a well-type, NaI crystal, scintillation counter. Counting geometry was maintained by using glass vials containing 2 ml of solutions or approximately 2 cc of dried bone chips. Corrections were made for resolving time of the counter but not for absorption in the samples. Initial counting of Cr^{51} and Br^{82} standards, blood, plasma and bone was done within 4 hours of obtaining the samples. Blood and bone samples were counted again at 24-hour intervals until the curve of decay indicated the only remaining radioactivity was from Cr^{51}. Four or 5 more counts were made at intervals of approximately 5 days.

Calculations. Extracellular fluid chloride concentration was calculated from serum chloride concentration using a Donnan factor of 0.977 and the observed serum water concentration in both cats and rats. The estimated interstitial chloride of bone was defined as the chloride that would be present if total bone water was an ultrafiltrate of plasma. This is



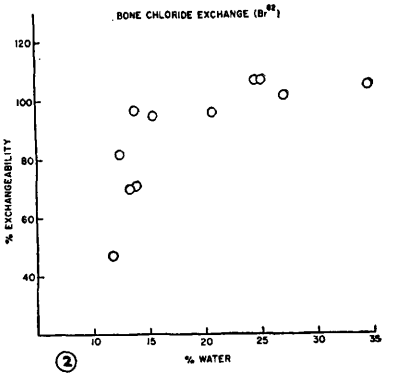


FIG. 1. Decay curve of KBr^{82} and Cr^{51} labeled red cells in aliquots of blood and bone from the same cat.

FIG. 2. Exchangeability of bone chloride determined with Br^{82} plotted against water content of dry fat-free bone.

based on the assumption that intracellular water of bone is negligible and that the water of the hydration shells of the crystals is freely permeable to the ions of extracellular fluid. Estimated Interstitial Chloride = G H_2O/g dry bone x $(Cl)_E$ where $(Cl)_E$ is the extracellular chloride concentration. Excess chloride was then defined as the value obtained by subtraction of estimated chloride from observed chloride content. The content of organic substances was estimated as the difference between total bone weight and the sum of ash and water contents. Separation of Br^{82} and Cr^{51} in blood and bone could be made by virtue of the fact that these isotopes have widely differing half-lives (Br^{82} 35.87 hrs, Cr^{51} 27.8 days). Fig. 1 shows a typical decay curve for aliquots of blood and bone in one cat. By inspection a regression line passing through the last points is drawn back

through the ordinate. This line has a half-life of 28 days, that of Cr^{51} and its intersection with the ordinate indicates counts minute due to radioactivity of Cr^{51} at the time of initial counting. The difference between this figure and the total counts minute is the radioactivity from Br^{82}. Assuming the venous hematocrit to be equal to the hematocrit of the blood in cortical bone, the blood volume of bone was calculated from the equation,

$$\text{Blood volume (ml/g bone)} = \frac{Cr^{51}\text{/g bone}}{Cr^{51}\text{/ml blood}}.$$

Since the Br^{82} ml blood was observed, blood chloride of bone was calculated as follows:

$$\frac{Br^{82}\text{/ml blood} \times \text{ml blood/g bone}}{SA^{\ddagger}} = \text{meq blood Cl/g bone.}$$

‡ SA = Br^{82}/meq. Cl.

Exchangeability of bone chloride was calculated as follows:

$$\text{Exchangeability (\%)} = \text{SA bone/SA serum} \times 100.$$

Results. Table I shows the observations on chloride of cat bone. The observed chloride content increased with increasing water content and decreasing body weight as did the estimated interstitial chloride content. However, an excess of chloride, above that calculated to be present in the interstitial fluid was observed in all bones. It is of interest that this excess of 1.53 meq/100 g fat free dry bone remained remarkably constant. The contents of ash and organic substances were noted to vary inversely within narrow limits.

Table II shows the contribution to the observed bone water and chloride contents by water and chloride calculated to be in blood of the marrow free cat bones. The small numbers of data do not warrant the assumption that this contribution increased with increasing bone water content.

Exchangeability of cat bone chloride as determined with Br^{82} is plotted against water content of bones in Fig. 2. The exchangeability was as low as 47% in bone with lowest water content (11.7 g %) and rose rapidly with increasing water content until at a level of approximately 15 g % it had reached 95%. The value of 100% indicates that the specific activity of serum was equal to that of bone, and variation around this value is evidence of the level of agreement of measurements of chloride by titration and using Br^{82}. A difference of 7% was reached when exchangeability was at its maximum indicating a close agreement.

Table III illustrates observations on bone chloride in rats. Though there was an increase in observed chloride and estimated interstitial chloride in these animals with increasing water content, the excess chloride was not constant, and present only in animals under 150 days of age. The value for excess chloride of 1.76 meq/100 g in weanlings was

TABLE I. Excess Chloride of Cat Bone (11 Animals).

Body wt (kg)	Water	Ash	Organic	ECF chloride (meq/l)	Observed chloride	Estimated interstitial chloride	Excess chloride
	(g %)*				(meq/100 g)*		
2.0	11.7	69.4	30.6	129	2.91	1.51	1.40
3.8	12.4	67.1	32.9	126	2.82	1.56	1.26
2.4	13.3	67.9	32.1	123	3.30	1.64	1.66
2.9	13.8	66.2	33.8	126	3.49	1.74	1.75
3.8	13.9	70.1	30.0	127	3.35	1.77	1.58
2.6	15.5	68.5	31.5	123	3.51	1.90	1.61
1.0	20.8	68.3	31.7	123	4.15	2.55	1.60
1.1	24.6	65.9	34.1	124	4.39	3.05	1.34
1.4	25.1	64.0	36.0	134	4.71	3.36	1.35
1.0	27.1	66.3	33.7	122	4.90	3.31	1.59
.9	34.7	63.2	36.8	129	6.20	4.47	1.73
					Avg		1.53
					Stand. dev.		.17

* Reference bone weights are those for fat free dry bone.

TABLE II. Percentage of Observed Bone Water and Chloride as Blood Water and Chloride (Cat).

Body wt (kg)	Bone: Water (g %)*	Bone: Blood (ml/100 g)*	Bone: Blood Cl (meq/100 g)*	Blood water, % of total bone water (%)†	Blood chloride, % of total bone chloride (%)
2.0	11.7	.48	.054	3.4	1.9
3.8	12.4	.51	.052	3.7	1.8
2.4	13.3	.22	.021	1.3	.6
2.9	13.8	.91	.093	5.5	2.7
1.4	25.1	2.14	.221	7.1	4.7

* Reference bone weights are those for fat-free dry bone.
† Calculated using observed water content of blood.

approximately the same as that found for cats.

Discussion. It is apparent that bone chloride content increased with increasing bone water content in both cats and rats. Subtraction of blood chloride from total chloride in cats does not eliminate this variation nor does subtraction of blood water from total water content eliminate variation in water content. The contribution of from 1.3% to 7.1% by blood water to total bone water is within the limits of 5-10% suggested by Neuman(5) for the amount present in the blood vessels, osteocytes, their lacunae and canaliculae.

It is also apparent that, in all but adult rats, there was more chloride present than would be estimated if all of the bone water had the same concentration of chloride as extracellular fluid. Since it has been estimated, by Eastoe and Eastoe(6), that 94% of the organic substance of cortical bone is collagen, and Nichols *et al.*(7) have found that dog tendon, which is comprised mostly of collagen, contains a chloride excess of 0.026 meq/g dry tissue, it is logical to postulate that the excess we observed was also located in collagen. However, in our experiment, only in cats did the ratio of excess chloride to organic substance remain constant. This ratio of 0.046 meq/g was the same as that found for weanling rats, but in this species, the ratio was not constant but decreased from the value of 0.047 meq/g in weanlings to zero in 150-day-old animals. It was also calculated that, since the ash and organic contents vary inversely in cat bone, ratio of excess chloride to ash also was constant at a value of 0.023 meq/g. Again in weanling rats this ratio was the same (0.028 meq/g) but decreased with increasing age in the same manner as the ratio of excess chloride to organic substances.

It was equally difficult to make any correlation of the ratios of excess chloride to water content since in cats the ratio decreased from 0.120 meq/g to 0.050 meq/g with an increase in water content from 12% to 35% while in rats the ratio increased from zero in bones containing 17% water to 0.034 meq/g in bones with 51% water.

The failure of any one of these ratios to remain constant in both species over the range of water concentrations and ages observed

TABLE III. Excess Chloride of Rat Bone.

Age (days)	Water (g %)*	Ash (g %)*	Organic (g %)*	ECF chloride (meq/l)	Observed chloride (meq/100 g)*	Estimated interstitial chloride (meq/100 g)*	Excess chloride (meq/100 g)*
157 (5)	16.5	74.1	25.9	112	1.73 ± .12†	1.84	−.11
111 (2)	22.7	72.4	27.6	110	2.67	2.49	.18
98 (4)	23.6	71.7	28.3	106	2.99 ± .08	2.49	.50
92 (3)	24.0	71.4	28.5	104	3.70 ± .31	2.51	1.19
82 (6)	27.1	71.2	28.8	108	4.19 ± .39	2.93	1.26
28 (5)	51.1	62.2	37.7	108	7.28 ± .58	5.52	1.76

* Reference bone weights are those for dry bone. † Stand. error. No. in parentheses refer to No. of rats analyzed.

makes it impossible to form, from these data, a satisfactory hypothesis concerning the location of excess chloride. It can only be stated that the fact that the exchangeability of the total bone chloride was less than 100% in presumably older cats with lower bone water concentrations suggests that the location of some of the chloride was less accessible in older animals. The very presence, however, of excess bone chloride makes the use of the chloride space as a measure of the extracellular fluid volume of bone unsatisfactory.

The accuracy of the chloride analysis is attested by the fact that measurements with Br^{82}, after maximum exchangeability was reached, were in close agreement with the titrometric analysis even though with Br^{82}, the only chemical measurement involved was plasma chloride. The observation that less than 5% of the observed bone chloride was present as blood chloride indicates that the samples were well cleaned of marrow and that this source of error was negligible.

Summary. 1) Observations on chloride content of bone of cats and rats revealed, in all but adult rats, that there was more chloride present than would be expected if the entire bone water content was extracellular fluid. This excess was constant at 1.53 meq 100 g of fat free dry bone in the cat over a wide range of water concentrations but, in rats, varied from a similar value in weanlings to the complete absence of an excess in 150-day-old animals. No satisfactory hypothesis could be made concerning the location of this fraction of the total bone chloride. 2) The accuracy of chloride analysis was attested by the maximum variation of 7% between titrometric determinations of chloride and determinations using Br^{82}. Studies with Cr^{51} revealed that the blood content of well cleaned bone samples contributes less than 5% of the observed chloride and less than 10% of the observed water content.

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X-Ray Photography of Human Cell Tumors in X-Ray and Cortisone-Treated Rats.* (24209)

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We wish to present a series of pictures, in reference to previously published data(1,2) showing the application of x-ray photography to the study of tumor development in treated weanling rats injected intraperitoneally with human cells. The cells implanted were from continuous cultures of HeLa, FL, and Detroit-6 strains. X-ray photography was a useful supplement to manual palpation for detection of solid tumors in the living animal. A permanent record of tumor growths in individual animals may be obtained in a series of x-ray photographs. The necessity for using a large number of animals for experimental purposes may be reduced by application of this method. This is particularly applicable when the wide variation in sizes of tumors, even under apparently uniform conditions is considered.

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