# ORGANIC AND BIOLOGICAL CHEMISTRY

[CONTRIBUTION FROM THE DIVISION OF PHARMACOLOGY, DEPARTMENT OF RADIATION BIOLOGY, SCHOOL OF MEDICINE AND DENTISTRY, UNIVERSITY OF ROCHESTER]

## The Uptake of Sodium and Potassium Ions by Hydrated Hydroxyapatite[1]

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RECEIVED NOVEMBER 2, 1955

Under carefully controlled conditions, hydroxyapatite was equilibrated with various mixtures of potassium and sodium chlorides. Potassium ion was found to penetrate only the hydration shell of the crystal while sodium, in addition, displaced calcium ions from the surfaces of the crystal lattice, apparently by a mole for mole non-equivalent exchange. These two physicochemical processes may account for the amounts of potassium and sodium observed to be present in fresh bone.

Sodium ion, the principal cation in the extracellular compartment of animals, is found in high concentrations in the skeleton. In fact, one-third to one-half of the total body content of sodium is associated with the mineral crystals of bone.[2] The physiological availability of this enormous, potential alkali reserve has been the subject of a number of recent investigations.[3-7] The chemical mechanisms underlying the skeletal fixation of sodium have received but little attention, however. One earlier report[8] attributed the presence of sodium in bone to an adsorption process as evidenced by the uptake of radiosodium by bone mineral *in vitro*. Chemical analyses for sodium were not performed. The skeletal content of potassium is very low[4] and, presumably, for this reason very few studies have been concerned with this ion.

It seemed possible that the sodium and potassium content found in fresh bone could be explained as the result of passive, physicochemical reactions such as ionic exchange. Certainly the bone mineral mirrors the composition of the solution in which it is placed[9] and the extracellular fluids bathing the bone crystals are high in sodium, low in potassium content. To test this hypothesis, alkali ion-bone mineral interaction was studied *in vitro* by suspending pure hydroxyapatite crystals in inorganic solutions of defined composition.

### Experimental

The use of ashed specimens of bone introduces uncertainties with respect to crystal size, high initial and variable sodium content, etc. Therefore, a well characterized, synthetic hydroxyapatite, "L-apatite"[10-12] was employed throughout these studies. This prototype bone mineral[9] contained less than 0.01% sodium. All other chemicals were C.P. grade unless otherwise indicated.

Equilibrations were carried out by high speed stirring of the solid and solution in a water-bath maintained at 37° as previously described.[13,14] The solutions contained sodium and potassium chlorides in varying proportions while the sums of their concentrations were kept constant to maintain a constant ionic strength. The solutions were buffered with 5.0 mmoles/l. of diethylbarbituric acid (Barbital—Mallinckrodt) at a *p*H of 7.38 ± 0.04 by the addition of approximately 2 mmoles/l. of KOH. Two grams of the dry hydroxyapatite was equilibrated with a liter of solution for 18 hours. Previous work has shown that solubility equilibrium is attained within this period[15] and preliminary experiments demonstrated that the sodium content of the solid phase reached a constant value within two hours. At the end of the equilibration period, approximately 30 ml. of the solution was withdrawn by suction through a very fine sintered glass filter to remove colloidal crystals[14] which prevent accurate analyses for calcium and phosphate. Aliquots of the filtered solution were added to 0.17 *M* ethanolamine (redistilled—Eastman Kodak Co.) and the calcium content determined by titration with disodium ethylenediaminetetraacetic acid (Versene—Bersworth), using the dye Eriochrome black-T (Hartman and Leddon) as the indicator.[16] Phosphate was determined by the method of Fiske and SubbaRow.[17]

The remainder of the solid-solution suspension was filtered through a sintered glass filter cup and centrifuged for two minutes at about 800 *g*. The solid was then transferred to specially designed cups[11] and centrifuged in a Servall ss-1 or ss-2 centrifuge at a force in excess of 7,000 *g*. After two hours centrifugation, the solid was dried and analyzed for sodium and potassium by flame photometry (Weichselbaum—Varney Universal Spectrophotometer).

**Centrifugation.**—The high speed centrifugation procedure is reported[11] to remove all mechanically held water leaving only the hydrated crystals. To confirm and extend the earlier study,[11] samples of hydroxyapatite were equilibrated with 0.16 *M* KCl in 0.005 *M* barbital and, after centrifugation at various speeds, the potassium content of each specimen after drying was determined flame photometrically. The results, presented in Fig. 1, are consistent with the view that mechanically held water is removed at forces in excess of 7,000 times gravity.

**Flame Photometry.**—The analysis of sodium by flame photometry gives large positive errors when calcium is present. As shown in Fig. 2, the increase in flame intensity is not nearly as marked when phosphate is also present. In solutions of calcium less than 5 millimolar containing phosphate (Ca/P = 1.67 as in the synthetic apatite), no measurable effect on flame intensity was noted. All solutions were therefore diluted prior to analysis for alkali metals to contain calcium at 5 mmoles/l. and phosphate at 3 mmoles/l. and the resulting solutions compared with stand-

(1) This paper is based on work performed under contract with the United States Atomic Energy Commission at the University of Rochester, Atomic Energy Project, Rochester, New York.

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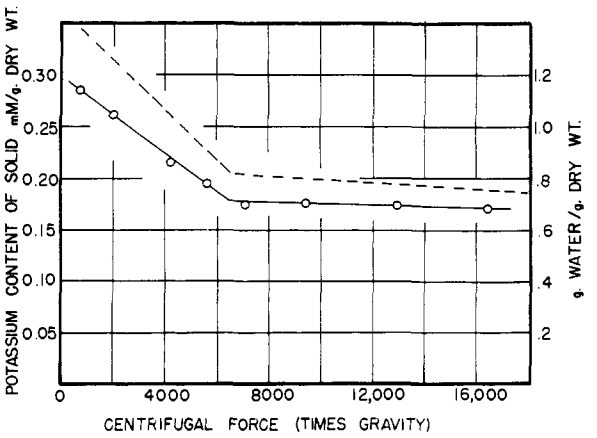


Fig. 1.—Data showing the relationship between the force of centrifugation and the amounts of water and potassium retained by the solid. Circles represent potassium content; the dashed line represents the water content taken from a previous publication.[11] Note that mechanically held solution appears to have been removed by centrifugation in excess of 6000 times gravity.

ards of similar composition. In addition, sufficient amounts of KCl were added to all solutions for sodium analysis to give a constant concentration of potassium and *vise versa* to minimize the effect of one alkali metal on the flame intensity of the other.

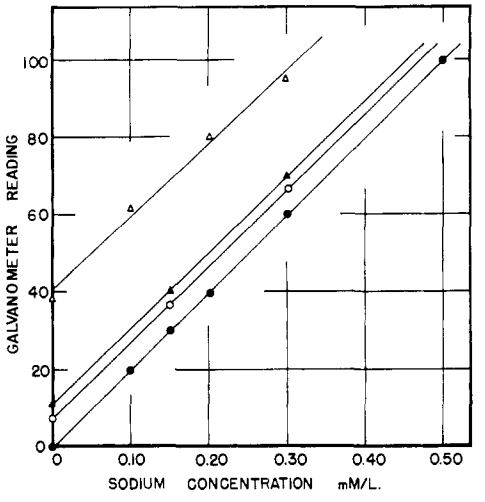


Fig. 2.—Effect of calcium and phosphorus on sodium standard curve measured by the flame photometer at wave length 589 $\mu$: ●, no added $Ca^{++}$ or P; ○, containing 50 mmoles/l. $Ca^{++}$ and 30 mmoles/l. P; ▲, containing 100 mmoles/l. $Ca^{++}$ and 60 mmoles/l. P; △, containing 50 mmoles/l. $Ca^{++}$ and no P.

## Results

**Sodium and Potassium Uptake.**—The first series of equilibrations was made at an ionic strength of 0.162. The results, given in Fig. 3a, show that potassium uptake by the crystal-hydration shell system is almost in strict proportion to the concentration of potassium in solution. Sodium was taken up by the solid phase to an extent greater than potassium and there was not a strict proportionality between solution concentration and solid phase content.

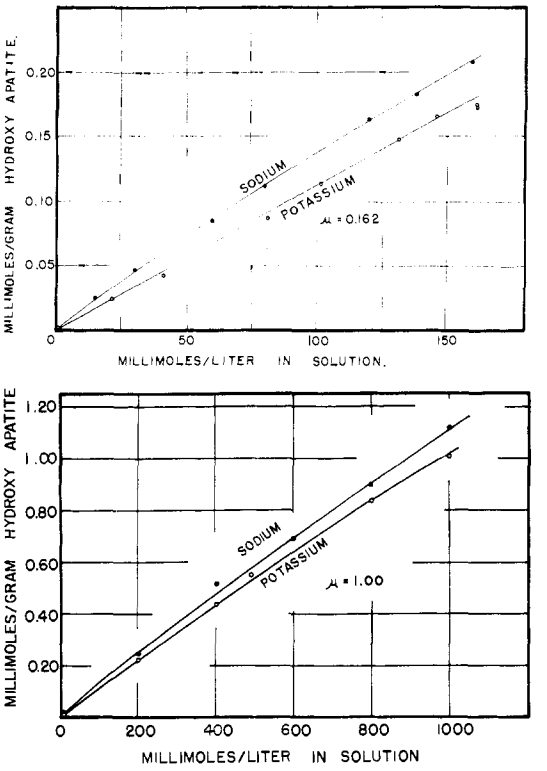


Fig. 3.—Uptake of sodium and potassium by hydrated hydroxyapatite.

A second series of equilibrations was performed at an ionic strength of 1.00. Again, as seen in Fig. 3b sodium was taken up by the solid to a greater extent than was potassium. At the higher ionic strength, however, neither ion was fixed in strict proportionality to the solution concentration.

**Reversibility.**—The reversibility of alkali metal binding was tested by the equilibration of samples of hydroxyapatite with four solutions always in the proportion of 2 g. of solid per liter of solution: A, 0.16 *M* NaCl; B, 0.16 *M* KCl; C, 1.0 *M* NaCl; D, 1.0 *M* KCl. The solids were filtered from the solutions and centrifuged. In two instances, the solids after treatment with A and C were re-equilibrated with B and D, respectively. Solid following equilibration with A was dried and reground before the second equilibration. The solid isolated from C was re-equilibrated immediately after centrifugation without drying. The results, given in Table I, indicate that the system is reversible within the limits of error of the methods of analysis.

TABLE I

TEST OF REVERSIBILITY OF THE UPTAKE OF SODIUM BY HYDROXYAPATITE

| Equilibrating soln. | Alkali metal content of solid Na, mmoles/g. | K, mmoles/g. |
|---|---|---|
| A, 0.16 *M* NaCl | 0.20 | .. |
| B, 0.16 *M* KCl | .. | 0.18 |
| A, then B | <0.01 | 0.18 |
| C, 1.0 *M* NaCl | 1.1 | ... |
| D, 1.0 *M* KCl | .. | 0.95 |
| C, then D | <0.01 | 1.0 |

**The Displacement of Calcium from the Crystals by Sodium Ion.**—Equilibration of hydroxyapatite

crystals with aqueous solutions has shown that NaCl as the bulk electrolyte dissolves more solid phase than does KCl. In particular, the final concentrations of dissolved or "displaced" calcium are much higher in aqueous NaCl than in equivalent solutions of KCl.[12] In these experiments, too, sodium ions appeared to displace calcium ions from the crystals resulting in higher concentrations of dissolved calcium. These data are given in Fig. 4.

## Discussion

It is helpful to review briefly some of the known facts concerning the apatite:solution system as a frame of reference for the interpretation of the data reported above. In an aqueous medium, the crystals possess a hydration layer[11] containing exchangeable ions[15] and the crystals themselves probably possess a net charge.[9] In such a system, a freely diffusible cation such as sodium or potassium could conceivably occupy several positions: a, in the bulk solutions; b, in the hydration shell; c, in the crystal surface; or d, a number of positions in the interior of the crystalline lattice. In the present studies the equilibration periods were too brief to permit penetration of the crystal interior[15] and, on theoretical grounds, it is unlikely that monovalent cations occupy interior positions (*vide infra*).

Under conditions where temperature, *p*H and ionic strength are all constant, the net charge per crystal and the volume of the hydration layer may be assumed to remain constant and, therefore, the amount of cation taken up by the solid due to diffusion into the hydration layer is given by the relation

$$f_{M'}[M'] = kf_M[M] \quad (1)$$

(hydration shell) (sol.)

or

$$[M'] = k\frac{f_M}{f_{M'}}[M] \quad (2)$$

where $f$ designates the activity coefficient and concentrations are indicated by brackets. This relation states that the amount of cation in the solid phase is directly proportional to the concentration of the cation in solution, *provided the cation does not exchange for other cations in the crystal surface.* The data given in Fig. 3 show that equation 2 describes the binding of potassium very well. At $\mu = 0.16$ the curve is linear within experimental error; at $\mu = 1.00$, there is a slight curvature which may be ascribed to small changes in activity coefficients as the mole fractions of potassium, $K^+/(Na^+ + K^+)$, varies from 0 to 1.

Since, under equilibrium conditions, the chemical potential in both solid and solution phases are equal, it may be assumed that the proportionality constant in equation 1 is equal to unity. With this assumption, the activity coefficient, $f_k'$, of potassium in the hydration shell can be calculated: 0.55 at $\mu = 0.16$ and 0.46 at $\mu = 1.0$. As one would expect, these values are considerably lower than in the bulk solution. It seems only reasonable that ion:ion interaction and ion:solvent interaction would be greater in a charge-oriented hydration layer than in ordinary solution.

Quite obviously equation 2 does not predict accurately the uptake of sodium ion. This is not surprising. It is a long established fact that bone mineral when boiled in non-aqueous alkali takes up great quantities of sodium but does not take up potassium. For this reason, the classical ashing procedures have always employed KOH.[18,19] Apparently, sodium ion can react with the crystal surfaces even in non-aqueous medium. For sodium ion to enter the crystal surface from an aqueous medium, calcium ion must be displaced and Fig. 4 shows that this displaced calcium appears in solution.

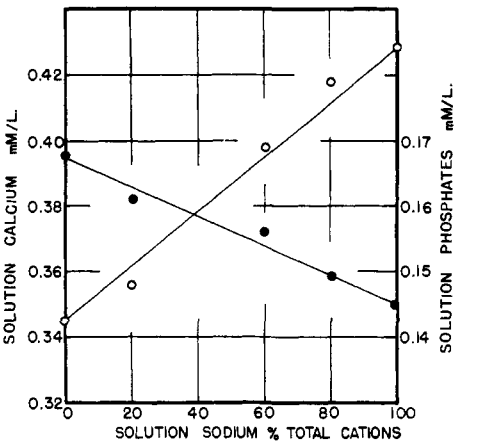


Fig. 4.—The displacement of calcium ions from hydroxyapatite by sodium ions at constant ionic strength; $\mu$ = 1.00: O, calcium; ●, total phosphates.

This displacement might occur on either a charge basis (2 monovalent sodium ions replacing one divalent calcium ion) or on a volume basis (one sodium ion of radius 0.95 Å. replacing one calcium ion of radius 0.98 Å.). Possibly a combination of space-charge limitations are governing the displacement reaction. Fortunately a partial answer to the mechanism of the exchange can be obtained by a mathematical analysis of the data.

The sodium ions entering the crystal surface may be represented by the total sodium content minus that sodium located in the hydration shell or

$$[Na]_{solid} = [Na]_{total} - [Na]_{hydration\ shell}$$

While $[Na]_{total}$ is observed, $[Na]_{hydration\ shell}$ must be estimated. If it is assumed that $[Na]_{hydration\ shell} = [K]_{hydration\ shell}$ under equivalent conditions, then

$$[Na]_{solid} = [Na]_{total} - [K]_{hydration\ shell}$$

and the sodium uptake resulting from *exchange* can be obtained by subtracting the potassium uptake curve from the observed total sodium uptake curve, *cf.* Fig. 3. From mass law, it follows that

$$[Ca]_{solid} + [Na]_{soln} = [Na]_{solid} + [Ca]_{soln}$$

or

$$[Na]_{solid} = k[Ca]_{solid}\left(\frac{a_{Na}}{a_{Ca}}\right)_{soln} \quad (3)$$

If two sodium ions replace a single calcium ion

$$[Ca]_{solid} + 2[Na]_{soln} = [Na_2]_{solid} + [Ca]_{soln}$$

or

$$[Na_2]_{solid} = k[Ca]_{solid}\left(\frac{a^2_{Na}}{a_{Ca}}\right)_{soln} \quad (4)$$

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In either case, linear relations can be expected only at low levels of sodium impregnation where the term $[Ca]_{solid}$ remains essentially constant. When the data are plotted according to equations 3 and 4 as in Fig. 5, a mole for mole exchange is indicated. Such a non-equivalent exchange can be expected either to reduce the positive charge on the crystals and/or result in a loss of anions associated with the crystals. It has, indeed, already been shown[15] that sodium impregnation sharply reduces the number of surface phosphate groups as measured by exchange techniques.

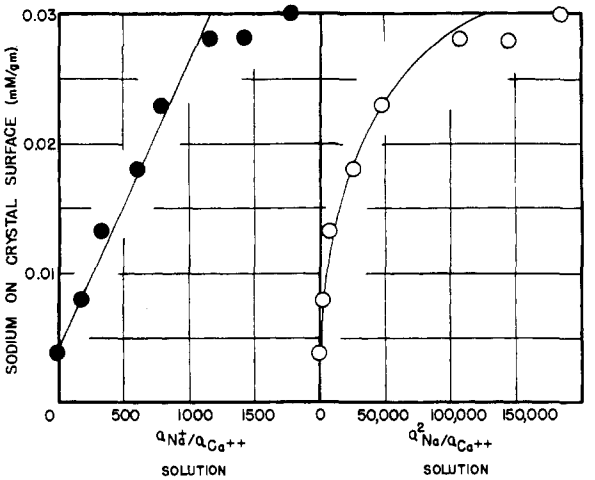


Fig. 5.—Indication of a mole for mole ionic competition between sodium and calcium with hydrated hydroxyapatite crystals; $\mu = 0.162$.

The data obtained in these studies *in vitro* cannot be extrapolated readily to the physiological state of bone *in vivo*. It is worth noting, however, that the sodium content of bone can be reasonably explained in terms of the hydration shell:crystal surface exchange concept. The crystals of bone are smaller than the crystals of apatite used in these studies (68 m.²/g.) and therefore present a greater surface area,[9,20,21] probably approaching 200 m.²/g. On the other hand the crystals in bone are not fully hydrated averaging only about 0.4 g. $H_2O$/g. apatite.[11] The sodium content of bone can thus be calculated from the results obtained here.

Crystal surface exchange = 0.04 mmole/g. $\times \frac{200 \text{ m.}^2/\text{g.}}{68 \text{ m.}^2/\text{g.}}$ = 0.12 mmole/g. (apatite or ash)

Hydration shell = 0.16 mmole/g. $\times \frac{0.4 \text{ g. } H_2O/\text{g.}}{0.8 \text{ g. } H_2O/\text{g.}}$ = 0.08 mmole/g.

Total predicted Na content = 0.2 m$M$/g. (apatite or ash).

Total predicted Na content = 0.2 m$M$/g. (apatite or ash). A number of analyses by flame photometry of various specimens of bone gave values for total sodium content varying between 0.2 and 0.25 mmole/g. of bone ash, in excellent agreement with the value predicted from the data obtained *in vitro*.

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# Composition of an Abnormal Protein Present in Tobacco Plants Infected with Tobacco Mosaic Virus[1,2]

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RECEIVED MARCH 18, 1955

An abnormal nucleic acid-free protein is present in tobacco plants infected with tobacco mosaic virus. Though lacking infectivity, this abnormal protein resembles the virus closely in appearance and in other physical properties. In the present study the abnormal protein and virus have been shown to contain the same amino acids. No quantitative differences in amino acid composition have been detected by column chromatography on hydrochloric acid hydrolysates of the two proteins. These results emphasize the close chemical relationship of the abnormal protein to the virus.

## Introduction

The presence of an abnormal protein, other than the virus in tobacco plants infected with tobacco mosaic virus, was reported by Commoner, Newmark and Rodenberg,[5] Takahashi and Ishii,[6] and Jeener and Lemoine.[7] Takahashi and Ishii[8] showed that this protein contained no nucleic acid, but yet could be polymerized into rod-like particles which under the electron microscope appeared to be identical in width and in general very similar to the virus. As part of an over-all study on the relationship of the abnormal protein to the virus, the amino acid composition of both were compared. The results are reported here.

## Experimental

Tobacco mosaic virus is readily obtained in high purity by means of well established procedures.[9] Isolation and purification of the abnormal protein is more difficult. By means of a procedure which we have outlined elsewhere,[10] the abnormal protein was obtained from a finely ground sus-

(1) Presented at the 125th Meeting of the American Chemical Society, Kansas City, Missouri, March, 1954.

(2) Aided by a grant from the National Foundation for Infantile Paralysis.

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(4) Department of Chemistry, Indiana University, Bloomington, Ind.

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