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Gas Adsorption and Surface Structure of Bone Mineral*

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Adsorption techniques have been applied to anorganic bone. Weight loss as a function of degassing temperature has been correlated with surface areas as determined by B. E. T. nitrogen adsorption. Pore size distribution in the bone mineral has been estimated on the basis of adsorption experiments and by use of the mercury porosimeter. From these results, as well as from the results of adsorption calorimetry, we suggest a model in which partially discrete and more or less randomly oriented crystals of submicroscopic dimensions are welded together by ionic forces and by adsorbed water bridging the grain boundaries in certain areas of contact. This model seems consistent with previously proposed models based on other experimental evidence such as X-ray and electron microscopic data.

A program of adsorption studies on anorganic bone has been conducted for several years in the Amherst Laboratory and in part at Carleton University. Various aspects of the work have been described in several publications (Dry and Beebe, 1960; Holmes and Beebe, 1961; Gale and Beebe, 1964). In this paper we shall discuss the effects of degassing temperature on the specific surface area. We shall give data on the pore size distribution of the bone mineral and present a possible model for its submicro crystal morphology.

Neuman and Neuman (1958) discuss in some detail the complexity of the problems with which investigators are confronted in attempting to characterize bone mineral with respect to both chemical composition and crystal morphology. The above authors point out that the tiny crystallites in bone, which are of submicroscopic dimensions, can be studied effectively by line broadening of X-rays and especially by means of the electron microscope. They cite the work of Finean and Engstrom (1953) and of Robinson and Watson (1952) as applications of these methods. They also discuss the work of Ascenzi (1955), who concludes from his electron microscopic studies that bone mineral is a continuous phase rather than an aggregation of more or less discrete crystals.

Neuman and Neuman summarize the present position in the following paragraph: "From the present status of information on the crystals of bone mineral, it seems reasonable to suppose that some of the divergence among investigators is caused by experimental artifacts. Some may also come from the examination of specimens in different stages of maturation. Thus a section beginning to mineralize would be expected to show collagen fibers associated with small, isolated, fragmentary crystals. As mineralization continued to the exclusion of interstitial water, the growth of crystals within the fibers and epitactic overgrowth of preexisting crystals would give the appearance of an almost continuous mineral phase."

The techniques of gas-solid adsorption have long been applied to the investigation of solid surfaces, especially those of catalysts, pigments, and porous adsorbents such as charcoal and silica gel. By these techniques it has been possible to deduce such properties as specific surface area, pore size distribution, and state of the adsorbing surface, whether energetically homogeneous or heterogeneous and whether polarizing or nonpolarizing. Rather extensive adsorption studies have been made on certain synthetic hydroxyapatites and bone chars (Barrett *et al.*, 1951a,b, 1952). With such experiments in mind it seemed to us that information supplementing the electron microscope and X-ray work might be obtained from adsorption studies on anorganic bone.

In order to bring the bone mineral into a reproducible state for the adsorption work, it is desirable to heat the sample in vacuo at 450°, usually for 24 hours. By this procedure virtually all adsorbed water can be removed without drastically altering the hydroxyapatite structure within the crystals, although there is some evidence for crystal growth during this heating process (Robinson and Watson, 1952). We feel that any crystal growth, although it would alter the extensive properties of the crystals, would not alter the intensive properties of the crystal surfaces. Thus a surface site might well possess the same energy for adsorption whether this site resided in the face of a crystal having a width of 60 Å or of 300 Å.

From the point of view of an interest in the bone structure *in vivo*, it is unfortunate that the bone mineral must be subjected to such drastic treatment in preparation for the adsorption studies. We feel, however, that the morphology of the dehydrated bone mineral would still bear a reasonably close resemblance to that in the parent bone. The point should be stressed

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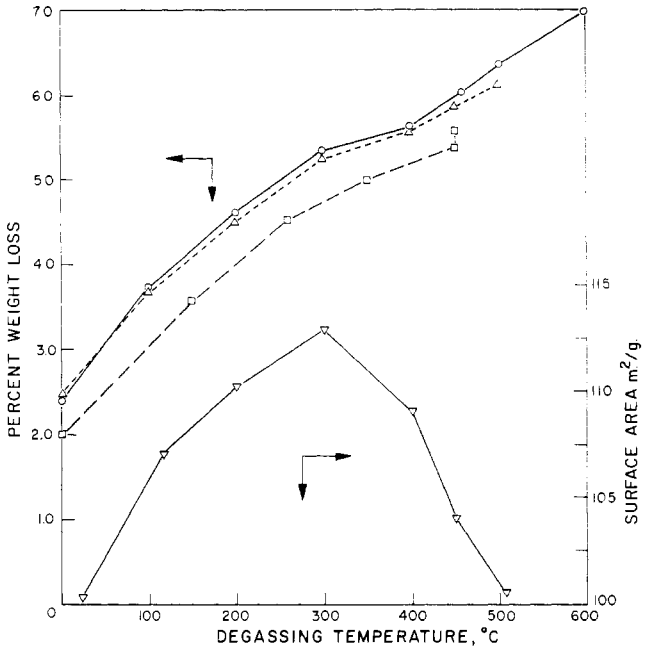

FIG. 1.—Percentage weight loss and surface area of bone mineral as a function of degassing temperature: ▽, average of several determinations of surface area; ○, △, □, runs 1, 2, 3, respectively, for percentage weight loss.

here that a quantitatively reproducible adsorbing surface can be obtained in this way (Holmes and Beebe, 1961, p. 295). The work of Dallemagne's group (Dallemagne and Herman, 1961) has shown that there is some formation of pyrophosphate when bone is heated to these temperatures. It is thought by Dallemagne that this pyrophosphate is "dissolved" in the apatite phase and there is no apparent change in the apatite specific surface.

On the basis of the adsorption work described in the present publication we have suggested a model for bone mineral structure which seems to be helpful in resolving the apparently discordant concepts of discrete crystals (Robinson and Watson, 1952) on the one hand and a continuous phase on the other (Ascenzi, 1955).

EXPERIMENTAL

Apparatus.—The volumetric gas adsorption apparatus used in measuring the nitrogen adsorption isotherms was of standard design used in many laboratories (Young and Crowell, 1962; Emmett, 1954) for surface area measurements (Brunauer *et al.*, 1938; Joyner, 1949). The mercury porosimeter was an instrument similar in construction to that described first by Washburn (1921, 1922) and later by Ritter and Drake (1945). The McBain balance used by us in the gravimetric method of studying nitrogen adsorption and in some of the experiments on dehydration of the bone mineral has been widely applied to adsorption work. This type of balance was first developed by McBain and Bakr (1926) and has more recently been used by many other authors (Boyd and Livingston, 1942; Bartell and Dobay, 1950; Dacey and Thomas, 1954). In some experiments we also used a procedure whereby the adsorption bulb was connected by a ground joint so the bulb could be weighed on a conventional analytical balance after various adsorption and evacuation cycles.

Bone Mineral.—The bone mineral used in this work was anorganic bovine femur supplied by the Armour Research Laboratory, designated as Ossar No. 33–43,

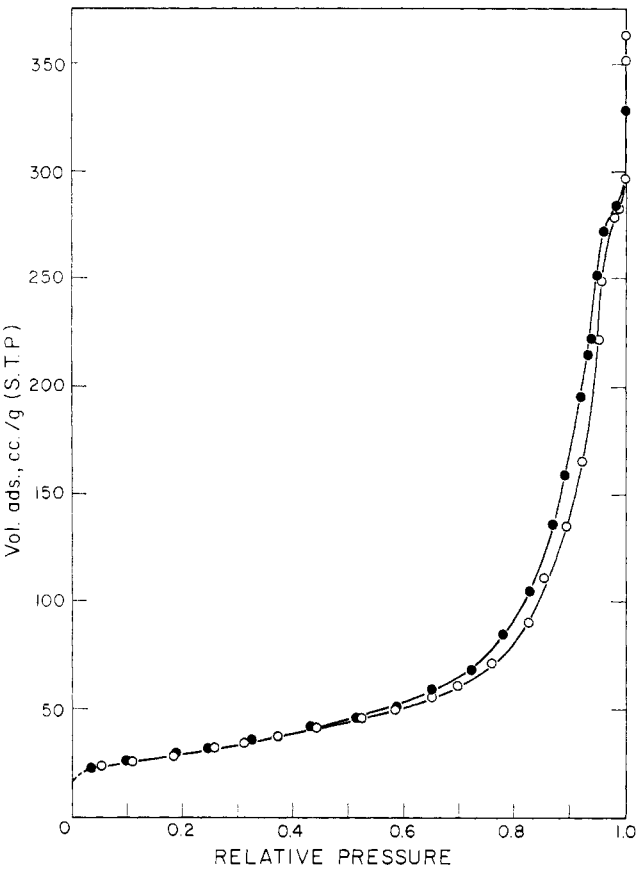

Fig. 2.—Typical isotherm at −195° for the adsorption of nitrogen on bone mineral sample outgassed at 450°. ○, adsorption; ●, desorption.

and described in an earlier publication (Dry and Beebe, 1960). It had been prepared from beef bone by extraction with ethylenediamine (Losee *et al.*, 1956; Losee and Hurley, 1957) and contained small quantities of residual carbonaceous materials as evidenced by slight darkening on heating above 300°. Because of the hygroscopic nature of this material there is a fundamental problem in even designating the weight of a given sample since the initial weight of the raw sample depends on the prevailing humidity of the atmosphere and since the extent of dehydration is dependent on the temperature and time of outgassing.

RESULTS

Changes in Weight and Surface Area on Degassing.—Because of the problems arising from the hygroscopic nature of the bone mineral, we have made a rather extensive investigation of the weight losses and changes in surface area on degassing at a series of temperatures.

The results of a typical set of measurements are shown in Figure 1. It is seen here that the weight of the sample decreases with rising temperature of degassing over the whole temperature range studied. There is a decrease in the slope of the weight loss versus temperature curve in the region of 350–450° which in part justifies our use of 450° evacuation temperature to produce what we have called in other papers the "bare" surface. The above data on bone mineral are in qualitative agreement with the reported results of Barrett *et al.* (1951a) for synthetic hydroxyapatite.

It is also evident from the data of Figure 1 that the specific surface area undergoes an increase of about 15% with increasing degassing temperature up to 300° but decreases for successively higher degassing tempera-

tures up to 500°. For temperatures much above 600° there is a sharper drop in specific surface area reaching a low value of about 10 m^2/g at 800° (not shown in Fig. 1) with a change in crystal structure of the material.

It seems reasonable that the increase in surface area for outgassing temperatures up to 300° is due to the opening up of certain small pores to nitrogen adsorption which were initially blocked by the presence of rather strongly bound adsorbed water. The existence of small pores and the fact that they can be covered up or restricted by the presence of water has been demonstrated in the calorimetric work of Dry and Beebe (1960) and of Holmes and Beebe (1961) and this is discussed below. The decrease in area observed upon degassing to temperatures above 300° may be caused by sintering becoming more important than the loss of water. Probably this sintering is due to a partial collapse of capillaries or grain boundaries in the apatite structure.

From these experiments on dehydration with the accompanying change in surface area, it is evident that rather close control of the outgassing temperature is necessary in order to obtain a reproducible surface for adsorption studies. To a certain degree, the time of outgassing is also important. We have selected 450° for the outgassing temperature and the experiments described below are based on a surface so prepared. While it is unfortunate that we must select arbitrary conditions for outgassing, this procedure is necessitated by the very complex nature of the bone mineral under study.

Rehydration of the Dehydrated Surface.—In the research cited above (Dry and Beebe, 1960) a rather detailed investigation was made of the process of rehydration of the dehydrated bone mineral, and in particular the energy of binding of water to the hydroxyapatite surface has been determined by means of calorimetry. The conclusions from this work may be summarized as follows: (1) The amount of water taken up at room temperature by the dehydrated bone mineral corresponds to about two monolayers if at a partial pressure of water vapor of 0.5. (2) The heat of adsorption of water runs from 20 to 14 kcal/mole in the first monolayer and from about 14 to 11 kcal in the second layer. From these data we may conclude that the first monolayer is strongly bound to the hydroxyapatite surface, probably by hydrogen bonding. As the second layer is filled, however, the heat values approach the heat of vaporization of water, thus indicating a binding energy only slightly in excess of that for water molecules to a liquid-water surface.

It is significant that two monolayers of water on a surface of 100 m^2/g specific area would represent about 0.05 g of water per gram of adsorbent, if we take the molecular area of adsorbed water to be 11.5 $Å^2$. This would be a 5% increase in weight due to hydration at a relative pressure of water of 0.5, which is in good agreement with the observed weight loss on dehydration. In making this comparison, however, we must bear in mind that the anorganic bone is an impure hydroxyapatite and that the initial "dehydration" process may thus involve the loss of some volatile material other than water, e.g., CO_2 (Dry and Beebe, 1960).

Pore Size Distribution.—The pore size distribution of the bone mineral was determined both from adsorption experiments and from mercury porosimeter data.

The isotherm for the adsorption of nitrogen at −195° on a sample outgassed at 450° is shown in Figure 2. The desorption branch of this isotherm was analyzed by the method proposed by Barrett *et al.* (1951b, 1952) and the results of the analysis are given in Figures 3 and 4.

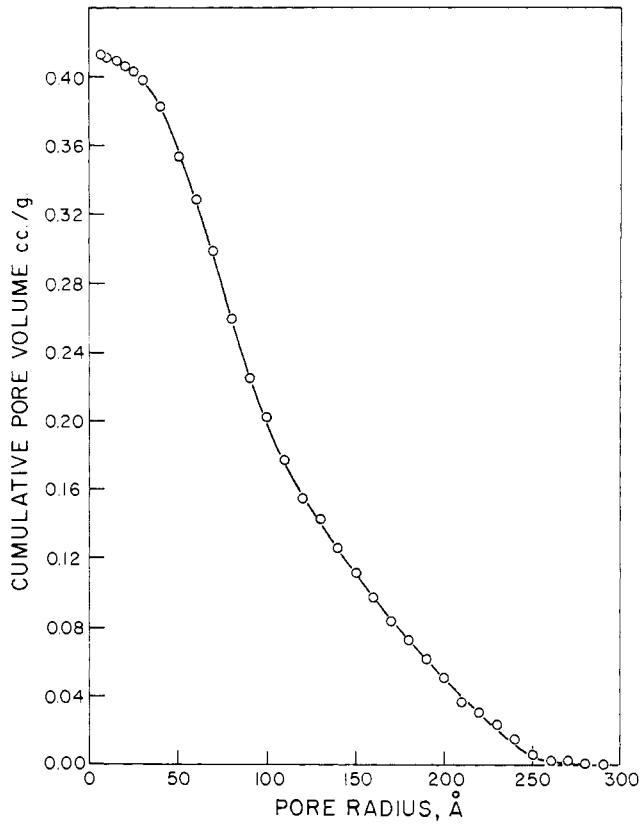

Fig. 3.—Cumulative pore volumes for a sample of bone mineral outgassed at 450°, calculated from nitrogen desorption isotherm data.

This type of analysis assumes that the hysteresis phenomenon is produced by capillary condensation, and yields the distribution of pore sizes in a model sorbent which would produce the observed hysteresis loop. The method is valid only for pores in which a liquid meniscus can form, and thus cannot give any information about pores whose radii are less than about 20 Å. It should also be pointed out that various approximations concerning the shape of the meniscus, the thickness of the adsorbed layer, its properties, and the shapes of the capillaries in the model sorbent have to be made in order to obtain a distribution. Thus these distributions have a somewhat formal significance, though they do give useful information on the structure of the sorbent. The results which we have obtained suggest that the majority of the pores contributing to hysteresis have radii between about 50 Å and 125 Å, but that there are pores in the sample having radii up to about 250 Å.

In previous publications we have reported the results of investigations of the adsorbed films of nitrogen on methanol-covered and water-covered samples of bone mineral. In the present work, isotherms of nitrogen at −195° were determined on these modified surfaces and analyzed to obtain pore size distributions. If the distributions for these modified surfaces were plotted together in Figures 3 and 4, they would be essentially coincident with the curves reported for the "bare" surface. Thus the presence of chemisorbed water or methanol makes little difference to the pore size distribution results.

The results of some mercury porosimeter experiments on two different samples of OSSAR (designated as No. 34 and No. 33–43) are shown in Figure 5. In these experiments considerable difficulty was experienced in attempting to duplicate the results. Thus we see that

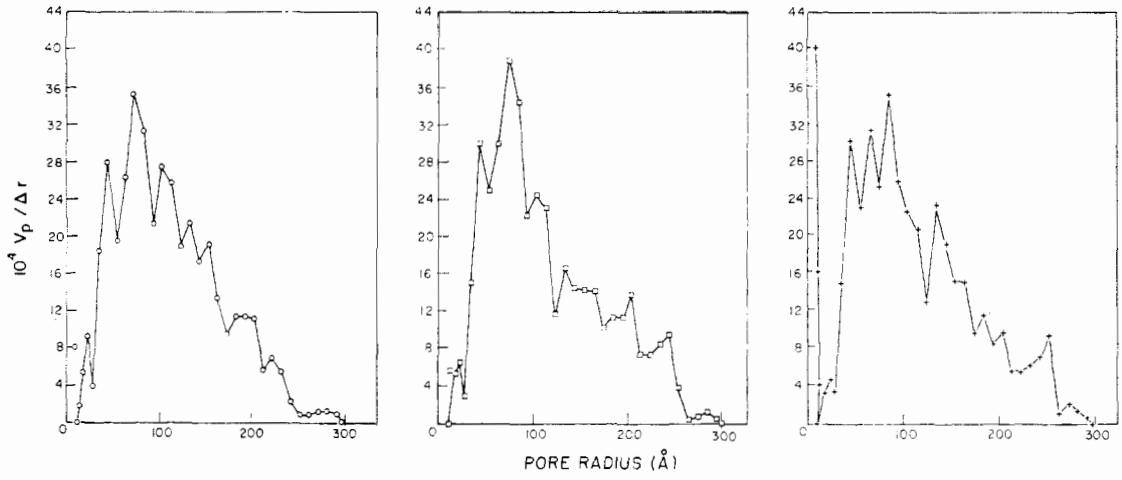

Fig. 4.—Pore size distribution for samples of bone mineral calculated from nitrogen desorption measurements: ○, bone mineral sample outgassed at 0°; □, bone mineral sample outgassed at 450°; +, bone mineral sample covered with chemisorbed methanol.

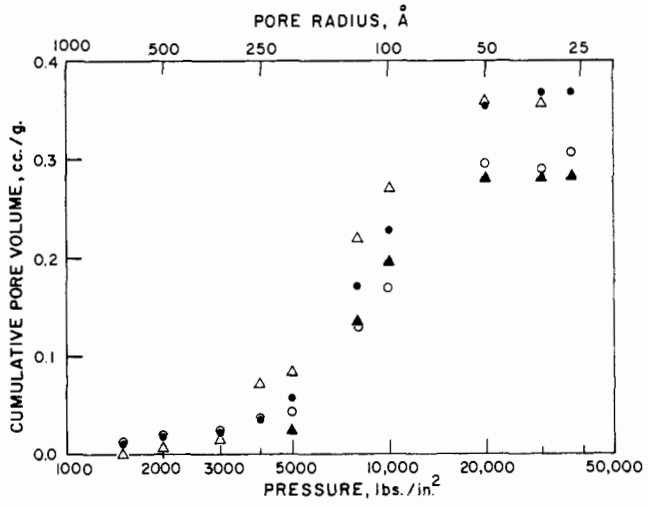

Fig. 5.—Cumulative pore volume distribution for samples of bone mineral calculated from mercury porosimeter data. Bone mineral not degassed before measurements made. △, ▲, duplicate runs on 10–20 mesh sample; ○, ●, duplicate runs on 20–40 mesh sample.

for both of the samples studied there was a 20 to 25% difference in the cumulative pore volumes obtained in successive experiments. It is probable that certain elements of the bone are being crushed as pressure is applied. Skeletal strength does vary within the sample, particularly if the particle size or shape is not uniform. (The sample actually contained particles of irregular shape ranging from 20 to 40 mesh.)

In principle, the distribution obtained from the porosimeter data should include pores whose shape is such that they do not contribute to adsorption hysteresis (e.g., cylindrical pores with one closed end). The presence of pores in which hysteresis does not occur will generally lead to a narrowing of the adsorption hysteresis loop and to an increase in the slope of the intermediate portion of the adsorption branch of the isotherm between the "B" point and the onset of hysteresis. However the lack of reproducibility in the porosimeter data makes any conclusions about such pores difficult. Nevertheless the porosimeter results are in qualitative agreement with those from the adsorption experiments.

Pores which are sufficiently large to admit adsorbate molecules but which are so small that no liquid meniscus can form in them will not contribute to the adsorption hysteresis nor will they be detected by mercury porosimeter experiments. Owing to their enhanced adsorption potential, they will be filled with adsorbate at low relative pressures and will contribute to the initial rapid rise in the adsorption isotherm. The decrease in surface area observed when a preadsorbed layer of water or methanol is present is probably caused by the blockage of these pores by the chemisorbed species. Additional evidence for pores of this size can be obtained from adsorption calorimetry.

In the calorimetric studies with methanol and in particular with water at 23° on the 450°-outgassed bone mineral surface (Dry and Beebe, 1960), an anomalous nonequilibrium state was observed which suggested a slow diffusion process into the adsorbent. In the heat measurements at −195° with nitrogen as adsorbate (Holmes and Beebe, 1961) an anomaly was observed which was somewhat different although probably due to the same underlying causes. Although this effect has been discussed in detail elsewhere, we may say here that, as successive increments of nitrogen were added to build up the first monolayer on the bone mineral surface, we observed an initial rapid temperature rise in the calorimeter which was followed by a much slower exothermal process. We attribute the initial rapid temperature rise to physical adsorption of nitrogen on easily accessible energy sites on the surface, and the subsequent slow evolution of heat to the transfer of nitrogen molecules from these easily accessible sites to less accessible sites of higher adsorption potential. Similar anomalies have been reported and discussed in earlier publications (Beebe and Dowden, 1938; Garner and Veal, 1935).

It seems plausible to assume that this slow transfer is due to a migration of the nitrogen molecules into *grain boundaries* between contiguous hydroxyapatite crystals. That these pores or grain boundaries are little wider than small molecular dimensions is supported by the observation that the slow diffusion process for nitrogen is virtually absent if we pre-adsorb a monolayer or two of chemisorbed methanol, presumably owing to the blocking of the pores by no more than two chemisorbed layers of methanol molecules.

A Model for the Crystal Morphology of Anorganic Bone.—It is well established that crystallites of the order of 50 by 200–300 Å are to be found in whole bone (Finean and Engstrom, 1953; Robinson and Watson, 1952). There seems to be no good reason to

believe that these crystals are greatly altered during the process of leaching out the organic matter and the subsequent dehydration at high temperature in preparing the bone mineral for adsorption studies. In fact, we have positive evidence for the stability of the interlacing anorganic bone mineral structure toward heat because of the small effect of heating on the specific surface area and pore size distribution (see Figures 1 and 4.)

From these adsorption studies we may conclude that the bone mineral has a porous structure with the major fraction of pore radii of the order of 50–125 Å and a smaller fraction or radii 125–250 Å. We suggest that these "pores" are the voids in an agglomerate of more or less randomly oriented crystals. It is reasonable that the dimensions of the voids should be of the same order of magnitude as those of the crystal units in the agglomerate mass.

As a first approximation to bone structure we may suggest the analogy of a pile of bricks thrown randomly together. This simple model would not account for the fact that anorganic bone, even after dehydration at 450°, does maintain its gross structure rather than falling apart into a mass of discrete submicroscopic crystals. For a somewhat more sophisticated model we might shave off certain corners or edges of the bricks so that they fit more closely together. The grain boundaries postulated earlier would be then represented by the areas (rather than points) of contact of the bricks.

In the case of the dehydrated anorganic bone we might still expect some interionic attraction across these grain boundaries. This attraction would be greatly increased upon hydration of the bone mineral since here we would have one or more hydrogen-bonded water molecules helping to bridge the gap across the grain boundaries. Of course in whole bone the strength of the whole structure is further greatly enhanced by the reinforcing effect of the interlacing collagen fibers which in turn are probably bonded to the inorganic matrix by hydrogen bonding either directly (Neuman *et al.*, 1953) or via intervening water molecules.

In the introduction we have cited a statement from Neuman and Neuman which implies divergence in the views of different investigators, especially as regards the concept of discrete crystallites of hydroxyapatite in bone mineral in contrast to the concept of a continuous phase of the mineral. It seems to us that the model we have proposed, which is based on evidence from the gas adsorption studies, tends to resolve this divergence. The high specific surface area of the anorganic bone can best be accounted for on the basis of submicroscopic crystals of the dimensions found by the electron microscope and by X-ray studies. On the other hand, a certain amount of intercrystalline bonding, perhaps by hydrogen-bonded water molecules, "would give the appearance of an almost continuous mineral phase."

Neuman and Neuman cite the results of kinetic studies which appear to indicate a stepwise process in the exchange of phosphate or of calcium ions between the bulk solution and a submicrocrystalline synthetic hydroxyapatite (apatite-L). To explain this stepwise behavior, they suggest a transfer of the phosphate or the calcium via a "hydration shell" from the bulk solution to the crystal surfaces followed by a further transfer from the crystallite surfaces into the interior crystal lattice. If one is to extrapolate from the behavior on synthetic hydroxyapatite to that on bone mineral, then a similar mechanism is implied for the latter adsorbent.

In view of our evidence for both a coarse pore structure of bone mineral (100–300 Å) and for very fine pores (perhaps <10 A), we suggest a somewhat modified version of the ion transfer mechanism put forward by Neuman and Neuman.[1] It seems to us that we could equally well account for the observed stepwise behavior in ion exchange by substituting, for the concept of a hydration shell around discrete crystals, a concept of a solution held in the coarse pores between the crystals of the hydroxyapatite. Furthermore the slowest process observed in ion exchange may be due in part to diffusion into the very narrow pores of grain boundaries rather than into the body of each of the individual crystals.

Neuman and Neuman (p. 59) postulate that the solution remaining on the hydroxyapatite after centrifugation at $30{,}000 \times g$ is held as a hydration shell. We suggest that the solution so retained may be held in the intercrystalline pores or voids of the apatite. It is cited that, in the centrifugation, "every crystal binds a hydration shell 1.9 times its own volume!" It is easier for us to accept the idea that this large volume of bound water is held largely in pores rather than in a hydration shell of the order of 100 Å thick.

Acknowledgment

We are indebted to Mr. Gerard Dubé of Carleton University who checked some of the weight loss and surface area measurements, and to Dr. B. I. Parsons, Bureau of Mines and Technical Surveys, Ottawa, who furnished the mercury porosimeter data.

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[1] We are aware that we are suggesting the use of a model for bone mineral to help explain phenomena involving synthetic hydroxyapatite. We feel that this is justified because of the similarity in the surface properties of these two materials as evidenced by earlier work in the Amherst Laboratory (Dry and Beebe, 1960).

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The Absolute Configuration of α-Hydroxy-β-carboxyisocaproic Acid (3-Isopropylmalic Acid), an Intermediate in Leucine Biosynthesis*

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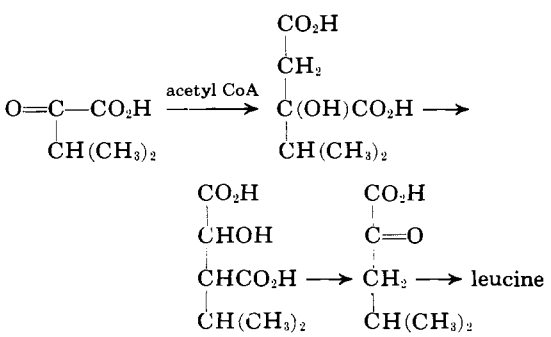
The absolute configuration of α-hydroxy-β-carboxyisocaproic acid isolated from a mutant of *Neurospora crassa* is shown to be *threo*-D_3-α-hydroxy-β-carboxyisocaproic acid. The *threo* configuration was deduced from nuclear magnetic resonance spectra of the *O*-acetyl anhydrides of the two racemates. Optical rotatory dispersion studies of natural α-hydroxy-β-carboxyisocaproic acid ethyl xanthate identified the α asymmetric center as a member of the D-series. Chemical reduction of the natural compound to L(−)-isopropylsuccinate confirmed the configuration of the β-carbon.

Recent work in several laboratories indicates that the following biosynthetic pathway to leucine is operative in several bacteria and fungi (Jungwirth *et al.*, 1961; Gross *et al.*, 1962, 1963; Calvo *et al.*, 1962; Strassman and Ceci, 1963):



This sequence of reactions is analogous to the formation of glutamic acid from oxaloacetic acid in the Krebs cycle. The absolute configuration of the isocitric acid isomer formed in the Krebs cycle has been shown to be *threo*-D_3 (Katsura, 1961).

The determination of the absolute configuration of α-hydroxy-β-carboxyisocaproate, a formidable task by classical organic techniques, was straightforward using nuclear magnetic resonance and optical rotatory dispersion. The configurational assignment was confirmed by chemical degradation.

Results

α-Hydroxy-β-carboxyisocaproate.—The synthesis of the two racemates of α-hydroxy-β-carboxyisocaproate (racemate A, mp 119–119.5°; racemate B, mp 122–122.3°) was described in an earlier report (Calvo *et al.*, 1962).[1]

Optically active α-hydroxy-β-carboxyisocaproate was isolated from *Neurospora crassa* strain D221a by a slight modification of the procedure of Burns *et al.* (1963). Seventy-five liters of culture filtrate was reduced in volume to 1 liter (temperature, *ca* 50°) and, after acidification to *p*H 1, the solution was extracted with three volumes of ether. The ether extract was concentrated to a 200-ml volume and was extracted with 100 ml of 10% $NaHCO_3$. After decolorization three times with 3 g portions of Darco G and acidification to *p*H 1, the solution was extracted three times with 100-ml portions of ether. Evaporation of the ether gave 10 g of a white crystalline residue. The residue was taken up in 5 parts of hot ethyl acetate and 10 parts of chloroform and, after standing overnight, 4.5 g of β-carboxy-β-hydroxyisocaproic acid (mp 164–166°) was removed by filtration. The mother liquors were evaporated to dryness *in vacuo*, the residue was taken up in an excess of ether, and an equal volume of *n*-heptane was added. Slow evaporation of the ether yielded 3 g of crude α-hydroxy-β-carboxyisocaproate. Recrystallization from the same solvent system gave 2 g of product, mp 130–136°. Both *Salmonella typhimurium* strains *leu* 120 and *leu* 128 responded to the isolated α-hydroxy-β-carboxyisocaproate in auxanographic tests (Jungwirth *et al.*, 1961); strain *leu* 120 also responded to β-carboxy-β-hydroxyisocaproic acid.

Racemic 2-Acetoxy-3-isopropylsuccinic Anhydrides.—The two synthetic racemates of α-hydroxy-β-carboxyisocaproate were converted to their *O*-acetyl anhydrides by addition of 4 ml of acetyl chloride to 1 g of racemate. After standing for 2 hours at room temperature, the derivatives were isolated by repeated passage of 50-μl

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[1] In the earlier report, the compound was referred to as 3-isopropylmalate; in this paper the name has been changed to α-hydroxy-β-carboxyisocaproate to conform with the usage of other workers (Gross *et al.*, 1963).