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STUDIES ON DEMINERALIZATION OF BONE*

1. The basic factors of demineralization

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Over 40 publications in scientific journals have appeared during the last 10 years reporting improved methods for the demineralization of bone; each of these methods claims to be superior to the existing methods of demineralization. One of two conclusions must be drawn from the large number of papers reporting successful results; either (1) bone demineralizes equally well by any method, or (2) it is impossible to judge the value of any method because of a lack of standards for comparison of results. All who have worked in this field agree that all methods of demineralization are not equally good; therefore, the difficulty must lie in the lack of comparative standards for evaluations.

A review of many experimental methods used by a number of technical innovators reveals this lack of standards. Since the repeatability of a technique is dependent upon duplication of the exact conditions of a method, the poor reporting of details makes it impossible, except by chance, to repeat the method and obtain the quality which the innovator describes. (For a complete bibliography on demineralization, see Gray[3].)

In general, most of the papers failed to give adequate information for repetition of method or evaluation of results in the following particulars:

1. The bone samples in the experiments vary widely in their surface area, weight, and proportions of compact and cancellous bone. Because the results are altered by the type bone used, lack of exact definition of the bone sample makes it impossible to compare the results.

2. The concentrations of the various reagents used for demineralization are not specified.

3. The conditions of demineralization are usually undefined. The temperature, frequency of solution change, agitation, and total amount of fluid used are omitted.

4. End point determinations of demineralization are vague. The most common method used was to probe the bone piece with a needle and judge from the resistance whether demineralization was complete.

5. The time necessary for demineralization is expressed frequently as plus or minus a period of hours or days.

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6. The basis of the histological opinion as to the relative excellence of a preparation is not given, only that the slide is judged as excellent, good, or fair.

It is apparent that a series of such difficulties would make duplication or comparison of techniques impossible.

The scope and purpose of this present work, therefore, is threefold:

1. To define standard conditions for the comparison of the efficiency of methods of demineralization.

2. To determine the basic factors which will be the limitations of any method of demineralization.

3. To determine whether varying the experimental conditions of temperature, agitation, type of acid, and molarity of acid bath shortens the time of demineralization.

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MATERIALS AND METHODS

I. STANDARD BONE: For all the experiments, sections of dense cortical bone from the shaft of one, air-dried, adult human femur were used. This bone previously had been analysed chemically to determine its average composition by taking 8 samples from various parts of the shaft; the individual determinations differed from each other by less than 1%. The average composition of the bone was:

Nitrogen	5%
Calcium	21%
Phosphorus	10%
Fat	11%
CO_2	3%
Water removed by heating at 125° C. for 4 weeks.....	18%

The amount of calcium and phosphorus for the standard bone was 0.81 mg per cubic millimeter of bone volume.

II. METHOD OF PREPARING BONE SAMPLES: Bone samples for an experiment were selected from adjacent parts of the shaft of the standard bone and given a rough shape by cutting with a band saw. The samples were then shaped by hand on medium and fine mill bastard files. Mill bastards were selected because of their hardness, which prevented metal fragments from contaminating the bone sample, and their deep grain, which removed the bone dust without its being ground into the minute canals within the specimen.

The bone pieces were measured with a vernier caliper, and the error in these measurements did not exceed ±0.1 mm. Surface areas were calculated from these measurements.

The weight of the bone sample was determined to the nearest milligram on a Roller-Smith torsion balance.

III. EXPERIMENTAL CONDITIONS: In all experiments, the following conditions were adhered to unless otherwise specified.

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1. Chemically clean polyethylene containers were used for demineralization. The tops of the containers were sealed with silicone stopcock lubricant.

2. Plastic instruments were used to remove the bone samples from the solutions.

3. 100 ml of solution of a known molarity were used.

4. The temperature of the demineralizing solution was maintained at 25° C. ± 1° C.

5. Chemical determinations were made upon aliquots of the complete demineralizing solution. New containers were used for each solution change to prevent contamination or loss of the solution through transfer.

6. All samples were agitated on a roller agitator at 85 rpm during the experiment. This machine is illustrated in Figure 1.

7. When the bone samples were removed to be weighed or X-rayed, they were blotted on bibulous paper by the same investigator each time; the weighings were made by the other investigator to keep errors constant. Repeated drying and weighings have shown this error to be not more than ± 1 mg.

8. The completeness of demineralization was determined by X-ray pictures or by clearing the bone piece in beechwood creosote.

9. Times were recorded in the experiments to the nearest minute.

IV. CHEMICAL TECHNIQUES: Four quantitative chemical determinations were made on each demineralizing solution. The individual determinations were made in quadruplicate.

1. Acid Standardization: The acid solutions before and after demineralization were titrated with standard sodium hydroxide solution using bromothymol blue, phenolphthalein, or bromocresol green as the indicator.

2. Nitrogen Determinations: Total nitrogens were determined by sulfuric acid digestion and steam distillation of digestion mixture as described by Koch and McMeekin[4]. Boric acid was used to collect the distillate. The distillate was Nesslerized (Koch and McMeekin[4]) and read at 10 minutes in a photoelectric colorimeter at 425 millimicrons.

3. Phosphorus Determinations: Phosphorus ion was determined by the method of Fiske and Subbarrow[2]. The technique was modified by increasing the amount of molybdate reagent 10 fold in place of the distilled water when making determinations on chelating acids because of the binding of the molybdate reagent by these compounds.

4. Calcium Determinations: Calcium was determined by titration of the calcium ion with di-sodium versenate* using purpurate as indicator as described by Elliott[1]. The technique was modified by precipitation of the calcium ion with ammonia water and ammonium oxalate and isolation by centrifugation before titration to eliminate interfering substances.

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EXPERIMENTS

Part 1: THE BASIC FACTORS IN BONE DEMINERALIZATION.

Experiment 1: The effect of variation in surface area upon time necessary for demineralization with weight a constant.

Pieces of the standard bone were shaped to form a bar and a cylinder having the same weight but different surface areas.

Data:	Weight	Surface Area	Surface area proportion of the smaller to the larger bone.
Bar	750 mg	4.64 sq. cm.	73%
Cylinder	753 mg	3.41 sq. cm.	

The bones were demineralized using the standard conditions (cf. Materials and Methods) in 0.5 molar hydrochloric acid for 8 hours. The bones were weighed every hour and the acid solution changed every 2 hours.

RESULTS:

The surface area of the smaller bone was 73% of the larger bone; the difference in weight between the samples was 3 mg. At the end of 8 hours of demineralization, the weight loss of the bar was 43 mg more than the cylinder, and the difference in the weight of calcium and phosphorus removed was 36 mg (Table I). Thus, the bone with the greater surface area was more demineralized than the bone with the lesser surface area.

Experiment 2: The effect of variation in weight upon the time necessary for demineralization with surface area a constant.

Pieces of standard bone were shaped to form a bar and a cylinder having the same surface area but different weights.

Data:	Weight	Surface Area	Weight proportion of the smaller to the larger bone.
Bar	639 mg	4.60 sq. cm.	57%
Cylinder	1129 mg	4.60 sq. cm.	

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The bone samples were demineralized using the standard conditions (cf. Materials and Methods) in 0.5 molar hydrochloric acid for 8 hours. The bones were weighed every hour and acid solution changed every 2 hours.

RESULTS:

The surface areas of the two bone samples were near identity, but smaller bone was 57% of the larger bone by weight. After 8 hours of demineralization, the difference in the weight lost by the two samples was 10 mg and a difference of 9 mg of calcium and phosphorus removed (Table II). Thus, bones with the same surface area but different weights showed almost no difference in weight loss or weight of mineral removal.

Experiment 3: The characteristics of mineral removal and diffusion.

A piece of standard bone was given the shape of a bar and demineralized in 0.5 molar hydrochloric acid for 6 hours; the experiment was stopped, and the demineralized matrix removed. The surface area and weight were again determined.

Data:	Weight	Surface Area
Original Sample:	1131 mg	4.81 sq. cm.
After Matrix Removal:	445 mg	2.87 sq. cm.

The bone was further demineralized under the same conditions for a second six-hour period. In both parts of the experiment, the bone was weighed and X-ray pictures taken of both front and profile views at half-hour intervals. Changes of acid solution were made every 2 hours. The depth to which mineral had been removed from the bone sample was measured on the X-ray negatives, and the surface area of the undemineralized portion was calculated.

RESULTS:

Measurements of the depth of demineralized matrix showed that demineralization proceeded simultaneously on all external surfaces of the bone about the same amount (Table III). Because of this, the form of the undemineralized portion did not change (Fig. 2).

There was a slightly greater depth of demineralization on the ends than on the sides of the sample. Most of the Haversian canals were open to the solution on the ends of the bone sample, and it has been found in other experiments that demineralization always proceeded a little more rapidly upon

those surfaces of the bone where the majority of the Haversian systems were open.

The more deeply the demineralizing agent penetrated into the matrix of the bone sample, the more slowly it penetrated per unit time (Table III). The depth of demineralization during the first two-hour period on the end of the sample was 0.54 mm; during the second two-hour period, the increase in depth was 0.26 mm; and during the third two-hour period it was 0.14 mm. Thus the increase in depth of demineralization for any period of time was about 50% of that for the previous time period.

Upon removal of the demineralized matrix in the second part of the experiment, the depth of demineralization repeated the pattern of the first 6 hours being 0.54 mm for the first 2 hours, 0.26 mm for the second 2 hours, and 0.18 mm for the final 2 hours (Table III).

This decrease in depth of bone demineralized each two-hour period was related neither to surface area nor to the weight of the bone piece. Rather, it was related to the increasing thickness of demineralized matrix between the solution and the undemineralized bone (cf. Fig. 2). This was demonstrated by comparing the increase in the depth of demineralized matrix during the last 2 hours before removal of the matrix and the first 2 hours after removal of the matrix. These depth increases were 0.14 mm and 0.54 mm. The only change of conditions between the two parts of the experiment was the removal of the demineralized matrix. All chemical substances, be they demineralizer or bone salts, must diffuse through the demineralized matrix. As the demineralized matrix accumulated, the demineralization slowed proportionally. This slowing of demineralization appeared to be the result of the diffusion process through the matrix.

The weight of calcium and phosphorus removed per two-hour period also decreased. During the first two-hour interval, 147 mg of calcium and phosphorus were removed; during the second two-hour interval, 57 mg; and during the third two-hour interval, 40 mg. The second six-hour period showed decreases in mineral removal which were proportional to those of the first period. Less mineral was removed in the second six-hour period than in the first and this reflected the difference in surface area of amount of mineral available to acid action (see Experiments 1 and 2).

Measurements from the surface of the bone piece to the sharp boundary of undemineralized bone in X-ray negatives (cf. Fig. 2) gave the necessary data to calculate the volume of bone demineralized. The amount of mineral removed from this volume

of bone is known from the chemical determinations. During the first six-hour period, 0.790 mg of calcium and phosphorus were removed per cubic millimeter of bone demineralized; during the second six-hour period, 0.787 mg of calcium and phosphorus were removed per cubic millimeter of bone demineralized (cf. Table III). The analysed standard bone (cf. Materials and Methods) showed an average calcium and phosphorus concentration of 0.81 mg per cubic millimeter.

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Discussion of Part I: THE BASIC FACTORS OF DEMINERALIZATION.

The basic factors in the demineralization of bone have been demonstrated by the first three experiments. These factors constitute the basic limitations of any method of bone demineralization.

1. The greater the surface area, the greater the volume of bone that was demineralized per unit time. Data from Experiment 2 showed that when the surface areas of bone pieces were equal, but the weights unequal, there was no appreciable difference in the amount of demineralization. In Experiment 1, where the weights of the bones were the same but the surface areas differed, there was considerable difference in demineralization. Therefore, if the surface areas of two bones are equal, the amount of demineralization will be equal regardless of the bone weights, and conversely, if the surface areas are unequal, the amount of demineralization will vary as the difference in surface areas.

2. Demineralization proceeded simultaneously on all surfaces of the bone at the same rate regardless of its surface area (cf. Table III, Depth of Demineralization).

3. Demineralization took place only at the interface between the demineralizing solution and the bone mineral. The amount of mineral removed in the experimental sample was about 3% (cf. Experiment 3) less than was found in the same volume of the analysed standard bone (cf. Materials and Mthods). The close comparison between analysed control bone standard and the experimental bone sample as well as the sharp boundary of undemineralized bone in the X-ray negatives strongly indicated that little or no demineralization took place except at the surface layer of the undemineralized part of the bone.

4. As demineralization continued, the process became progressively slower because of the increased diffusion distance through demineralized matrix. This was demonstrated by Experiment 3. This experiment showed that the rate of demineralization decreased as the thickness of demineralized matrix increased (cf. Table III, Depth of Demineralization); upon removal of the demineralized matrix, the rate of demineralization returned to its initial value and again decreased as the thickness of the demineralized matrix increased.

In this experiment, all factors were constant except the changes in the thickness of the demineralized matrix. It is evident that the demineralizing acid must diffuse through the matrix layer to reach the undemineralized bone surface, and that the bone mineral must diffuse out by the same route. The greater the diffusion distance, the longer the time necessary for these substances to diffuse. The slowing of demineralization was proportional to the thickening of the demineralized matrix. The

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slowing of demineralization then, was proportional to the increase in the diffusion distance through the demineralized matrix. Therefore, the progressive slowing of the demineralization process is due to the increase in time necessary for the various substances to diffuse into and out of the bone through the constantly increasing thickness of demineralized matrix.

5. During demineralization, the surface area of undemineralized bone was continuously decreased, thus decreasing the amount of bone mineral available to the acid because demineralization took place only at the mineral-acid interface. This was demonstrated by Experiment 3 in which 244 mg of mineral were removed in 6 hours from a bone whose surface area was reduced from 4.81 to 2.97 sq. cm.; in the same bone following removal of the demineralized matrix, a second 6 hours of demineralization gave 122 mg of mineral when the surface area was reduced from 2.87 to 1.35 sq. cm. When the surface area was reduced by about 50%, the amount of mineral removed was reduced by about 50%. In both instances, the depth of demineralized matrix was the same indicating that the rate of demineralization was the same for both.

It must be expected, therefore, that a smaller volume of bone will be demineralized as the process approaches completion because of the continual reduction in surface area. However, the increase in time necessary for demineralization is not due to this reduction in surface area, but to the increasing distance of diffusion; the rate per unit of surface area remains constant.

Part II: THE EFFECTS OF ALTERATION OF THE CONDITIONS ON DEMINERALIZATION.

The limitations described in Section I are unavoidable, but this does not preclude adjustment of the conditions of demineralization to obtain the greatest efficiency within these limitations. Two groups of conditions may be manipulated to obtain a greater efficiency:

1) Those factors which increase the rate of diffusion (e.g., temperature and agitation).

2) Those factors which increase the efficiency of the demineralizer (e.g., type and concentration).

Experiment 4: The effect of agitation upon the rate of demineralization.

Experiment 4A (8 hours demineralization): Two pieces of standard bone were shaped to approximately the same weight and surface area.

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Data:	Weight	Surface Area
Agitation Sample	575 mg	3.36 sq. cm.
Non-agitation Sample	573 mg	3.36 sq. cm.

Experiment 4B (69 hours demineralization): Two pieces of standard bone were shaped to approximately the same weight and surface area.

Data:	Weight	Surface Area
Agitation Sample	783 mg	3.92 sq. cm.
Non-agitation Sample	788 mg	3.93 sq. cm.

In both Experiment 4A and 4B, the conditions were the same except for the length of time the experiment was conducted. Experiment 4A was concluded after 8 hours of demineralization while Experiment 4B continued for 69 hours of demineralization. In both experiments, the samples were placed in 0.5 molar hydrochloric acid. The containers with the agitation samples were placed upon a roller agitator revolving at 85 rpm. The non-agitation samples were left undisturbed in their containers between weighings. The samples were cleared in beechwood creosote and the depth to which the mineral had been removed from the samples was measured. The times of weighing of the samples and the solution changes are recorded in Table IV.

RESULTS:

In both Experiment 4A and 4B, the surfaces of the non-agitated samples rapidly collected a layer of bubbles which eventually encased the samples in an envelope of gas. This gas envelope decreased the amount of contact between the demineralizing solution and the bone, and probably was the major cause of the slower demineralization of the non-agitated samples. The conditions were not constant because it was necessary to remove the gas envelope when the samples were dried for the periodic weighings. This gave a slight increase to the rate of demineralization for the non-agitated specimens.

Measurements of the volume of demineralized bone in Experiment 4A (8 hours demineralization) showed that the mineral was removed from about 32% of the volume of the agitated sample and from about 25% of the non-agitated sample. In Experiment 4B, a similar result was seen. The agitated sample was demineralized completely in 50 hours but the non-agitated sample after 69 hours demineralization still contained a small undemineralized core measuring approximately 4 x 1.5 x 1.5 mm.

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Further evidence of the difference between agitated and non-agitated samples was seen in the data (cf. Table IV). In Experiment 4B (69 hours demineralization) the agitated specimen lost 44% of its weight in 50 hours and was completely demineralized while the non-agitated specimen did not reach this percent weight loss until 69 hours of demineralization. The agitated specimen was demineralized at least 38% faster that the non-agitated specimen.

Similarly, in Experiment 4A (8 hours demineralization), the agitated specimen reached 21% weight loss in 4 hours while the non-agitated specimen did not reach this percent weight loss until 6 hours of demineralization. The agitated sample had demineralized to the same extent in about 33% less time than the non-agitated sample.

Thus, in general, the agitated specimens demineralized at least 30% more rapidly than the corresponding non-agitated samples.

Experiment 5: <u>The effect of variation in temperature upon the rate of demineralization.</u>

Four samples of approximately equal weight and surface area were prepared from the same part of the standard bone.

Data:

	26°C.	36°C.	46°C.	56°C.
Weight (mg)	586	587	585	587
Surface Area (sq. cm.)	2.80	2.80	2.80	2.80

The samples were placed in 0.5 molar hydrochloric acid and solution temperatures were controlled at ± 1°C. during the experiment. The bones were demineralized for 8 hours and weighed every hour; the acid solutions were changed every 2 hours.

<u>RESULTS</u>:

The data from this experiment are recorded in Table V. These data showed that raising the temperature of the demineralizing solution increased the amount of mineral removed per unit time, but above 36°C., various side effects occurred which were undesirable. This was seen if the mineral removed was expressed as a percent of the total weight loss for each 10°C. increase in temperature:

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at 26°C 81% of the weight loss was mineral removed.

at 36°C 78% of the weight loss was mineral removed.

at 46°C 73% of the weight loss was mineral removed.

at 56°C 66% of the weight loss was mineral removed.

While more mineral was removed at the higher temperatures, larger amounts of other materials were also removed (as much as 33% at 56°C). Chemical studies indicated these materials were primarily proteins and fats of the organic matrix.

Similarly, there was a decrease in efficiency of demineralization as the temperature increased because:

a temperature increase from 26° to 36°C. gave a 10% increase in mineral removed

a temperature increase from 36° to 46°C. gave a 4% increase in mineral removed

a temperature increase from 45° to 56°C. gave a 6% increase in mineral removed.

Thus, while more mineral was removed at the higher temperatures, the rate of removal was not proportional to the temperature increase.

A temperature around 36°C. appeared to be the most advantageous because this gave maximal mineral removal with minimal unwanted side effects.

Experiment 6: <u>The efficiency of various acids in demineralization.</u>

Sections of standard bone were prepared having similar weights and surface areas; the weights of the bone varied from 400 to 434 mg and the surface areas varied from 0.218 to 0.235 sq. cm. These bone samples were placed in 1 molar concentrations of hydrochloric, nitric, hydrobromic, phosphoric, trichloroacetic formic, lactic, acetic or citric acid. The bones were demineralized for 8 hours and weighed every hour. Acid solutions were changed every 2 hours. At the end of the experiment, the samples were cleared in beechwood creosote and the depth of demineralized matrix measured.

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RESULTS:

Table VI summarizes the relative rates of mineral breakdown and removal by the acids tested. These relative rates were based on the percent weight loss of the bones after 8 hours of demineralization. The total mineral removed by each acid is also summarized in Table VI.

When the percent weight loss, depth of demineralized matrix, and mineral removed were compared with the dissociation constant and solubility of the calcium salt of the acids, an almost perfect relation was found. The acids were grouped into three divisions. These divisions were according to the separations which occurred in the data. The following summary will show this more clearly:

Range of percent weight loss	Range of Mineral removed	Range of solubility of calcium salts of the acids at 25°C.*	Range of dissociation constants of the acids*
		GROUP I	
28-31%	99-120 mg	125-315 g/100ml HOH	$7 \text{ to } 9 \times 10^{-1}$
		GROUP II	
7-16%	31-62 mg	35-70 g/100ml HOH	$2 \text{ to } 10^{-1} \text{ to } 1.76 \times 10^{-4}$
		GROUP III	
1-4%	0-19 mg	0.25-17 g/100ml HOH	$1.38 \times 10^{-4} \text{ to } 1.76 \times 10^{-5}$

In the above relations, the acids with the highest dissociation constant and solubility of their calcium salts had the most rapid rates of demineralization (Group I). The lowest rates of demineralization were seen with those acids which had the lowest calcium salt solubility and dissociation constants (Group III).

*These figures were taken from Handbook of Chemistry and Physics, 31st Edition, Charles D. Hodgman, Editor. Cleveland, Chemical Rubber Publishing Company, 1949.

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A more detailed summary of the grouping of the individual acids may be seen below. The acid at the top of each column had the highest value in the category and the acid at the bottom, the lowest value in the category.

	Percent weight loss with acid	Depth of demineralized matrix with acid	Mineral removed with acid	Solubility of the calcium salt of the acid	Dissociation Constant of the acid
Group I	HCl	HCl	HCl	HNO_3	HBr
	HNO_3	HBr	HNO_3	HCl	HNO_3
	HBr	HNO_3	HBr	HBr	HCl
Group II	Cl_3COOH	H_3PO_4	Cl_3COOH	Cl_3COOH	Cl_3COOH
	H_3PO_4	Cl_3COOH	H_3PO_4	Acetic	H_3PO_4
	Formic	Formic	Formic	H_3PO_4	Formic
Group III	Lactic	Lactic	Lactic	Formic	Lactic
	Citric	Citric	Acetic	Lactic	Citric
	Acetic	Acetic	Citric	Citric	Acetic

The rate of breakdown of the mineral in the organic matrix in the bone is determined by the dissociation of the acid. The solubility of the calcium salt will determine, in part, the rate of removal by diffusion through the demineralized matrix. It is difficult to separate these two components experimentally because the dissociation constants of acids usually parallel the solubility of their calcium salts, Acetic acid, however, did not follow this general rule. The solubility of calcium acetate stands higher in the classification than does the dissociation of acetic acid (cf. above). The demineralization rate of acetic acid was the lowest of all the acids used, and it also had the lowest dissociation constant of all the acids in this experiment. According to the solubility of calcium acetate, this acid should rank in the group with trichloroacetic acid, not as the poorest demineralizing agent tested (cf. above). From this, it would appear that the solubility of the calcium salt of an acid was of no benefit if the mineral was not broken down in bone. Thus, it seems reasonable to conclude that the rate of demineralization is primarily determined by the rate of mineral breakdown which is related to the dissociation constant of the acid.

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Titrations ofthe acid concentrations at the end of the experiment showed the amount of acid utilized in the demineralization process. The maximal amount of acid utilized was 34% in the case of citric acid (cf. Table VI); the other acids averaged about 10% utilization during the 8 hours of demineralization. Since a 10% reduction in molarity only reduced the concentration from 1 to 0.9 molar, an adequate amount of acid was present for demineralization (cf. Experiment 7 for the minimal acid concentration for effective demineralization).

A measure of the efficiency of an acid in the removal of mineral from bone has been established by determining the number of millimoles of hydrogen used to remove 1 millimole of the mixture of calcium and phosphorus in bone. This ratio of hydrogen to mineral removed is recorded in Table VI. The data showed hydrochloric acid to be the most efficient in removal of mineral since 0.98 millimole of hydrogen removed 1 millimole of the mineral mixture. Hydrochloric acid was followed by trichloroacetic, formic, and phosphoric acids, all of which had a hydrogen to mineral removed ratio of less than 2:1. All the other acids had a ratio of more than 3:1 which was considered to be inefficient. Chemical determinations showed that the acids with a ratio higher than 3:1 had a higher nitrogen concentration in the solution following demineralization. It is suspected that the excessive amount of hydrogen was used in hydrolysing protein rather than removing mineral. This same phenomenon of high nitrogen removal from the demineralizing bones also has been noticed in the higher concentrations of trichloroacetic acid.

From this experiment, it has been concluded that the rate of mineral breakdown is a function of the dissociation constant of the demineralizing acid because, the higher the dissociation of the acid, the more rapid was the mineral removal. In all determinations, hydrochloric acid ranked first and was the most rapid demineralizing agent.

Experiment 7: <u>The effect of concentrations of an acid upon the rate of demineralization.</u>

Six samples of approximately equal weights and surface areas were prepared from the same part of the standard bone. One sample was placed in each of the following concentrations of hydrochloric acid and demineralized for 8 hours:

4 Molar

2 Molar

1 Molar

0.5 Molar

0.1 Molar

0.05 Molar

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The samples were weighed every hour and the acid solutions changed every 2 hours. At the end of the experiment, the bone pieces were cleared in beechwood creosote and the depth of the demineralized matrix was measured.

RESULTS:

At the end of 8 hours of demineralization there was no difference in percent weight loss or weight of mineral removed (cf. Table VII) with 4 and 2 molar hydrochloric acid. The weight loss of the bone sample in 1 molar acid was 89% of the weight loss of the bone in 2 molar hydrochloric acid, and the mineral removed in 1 molar acid was 92% of that removed in 2 molar acid. The mineral removal and weight loss with 0.5 molar hydrochloric acid was 71% of the 2 molar acid. Demineralization in 0.1 and 0.05 molar concentrations were of such low orders that they were considered to be ineffective.

A peculiarity was noticed in the demineralization with 0.1 and 0.05 molar hydrochloric acid. In these, the amount of mineral removed exceeded the amount of weight loss (Table VII). A subsequent experiment showed this result to be due to the absorption of water by the demineralized matrix of the bone since the differences in weight loss of the completely demineralized bones disappeared upon drying for 6 days at 125°C.

From these results, it was clear that increasing the molarity of the acid beyond 2 molar did not increase the efficiency of demineralization. Below 0.5 molar concentrations of the acid, demineralization was unduly prolonged. The optimal range of concentration for hydrochloric acid in demineralization was between 0.5 molar and 2 molar, the optimal concentration being between 1 and 2 molar.

Discussion of Part II: THE EFFECTS OF ALTERATION OF CONDITIONS OF DEMINERALIZATION.

In Section I of this paper, the inescapable limitations of any process of demineralization were described; Section II has been devoted to the determination of the optimal conditions to be used in demineralization within this framework of basic limitations. In the determination of the optimal conditions, four factors of demineralization were isolated and studied: The effect of agitation, the effect of increases in temperature, the relative value of several acids as demineralizers, and the optimal concentrations of an acid for maximal demineralizing efficiency.

By utilizing the optimal conditions of each of these factors, it was possible to reduce the time of demineralization by more than 50% as compared with the usual methods using the same reagents.

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Effect of agitation: Continual agitation of bone samples during demineralization increased demineralization about 30% (cf. Experiment 4). The rate of agitation is not important provided the bone sample is continually tumbling in the solution. The rate of 85 rpm was used in these studies because this rate insured the continual tumbling regardless of the size of the bone pieces used in the experiments.

Three reasons are given for the shortening of demineralization time by agitation. 1) Agitation prevents the accumulation of gas bubbles on the surface of the bone piece. This bubble layer limits the contact with the demineralizing acid and reduces the rate of demineralization. 2) Agitation prevents the bone piece from resting on the bottom of the container. When the bone piece rests on the bottom of the container, its area of contact with the demineralizing acid is limited. 3) Agitation insures an equal distribution of the bone salts and demineralizing acid in the demineralizing solution. The removal of mineral from bone is primarily a diffusion process, and in a static system, the salts may tend to accumulate at the surface of the bone; these salts are dispersed and replaced by acid during agitation.

Effect of increases in temperature: An increase in the temperature of the demineralizing solution from 25°C. (average room temperature) to 36-40°C. decreased the time of demineralization about 15% (cf. Experiment 5). Temperature increases above 40°C. showed a continued decrease in the time for demineralization, but the amount of mineral removed was not proportional to this decrease; in addition, the higher temperatures seemed to increase the amount of destruction of the organic matrix. An increase in temperature from 26 to 56°C. decreased the time of demineralization about 20%, but the destruction of matrix, as indicated by nitrogen determinations, was about twice that at 36°C. For the additional 5% decrease in demineralization time, the unwanted side effects were doubled.

Evaluation of some acid demineralizers: The rate of mineral breakdown in bone by an acid was related to its degree of ionization; acids with high dissociation constants were rapid demineralizers and acids with low dissociation constants were slow demineralizers (cf. Experiment 6).

Associated with the relation of acid ionization to rapid demineralization was the solubility of the calcium salt of the acid. The relative ranking according to calcium salt solubility usually paralleled the rank of the acid according to its dissociation constant. The acids with the highest rates of demineralization also had the highest calcium salt solubilities which insured a more rapid diffusion of the mineral out of the bone.

When qualifications of various acids according to the rapidity of demineralization, efficiency of action (as determined by the ratio of millimoles of hydrogen used to remove 1 millimole of mineral), and the minimal removal of nitrogen from the bone matrix were compared, hydrochloric acid ranked first in all categories.

Hydrobromic and nitric acids were rapid demineralizers, but they had the disadvantage of a relative high destruction of protein structures. Citric, lactic, and acetic acids had such low rates of demineralization as to be valueless.

Some methods reported have used salts with acids in demineralization because this mixture was believed to give more rapid and satisfactory results. Several salts were tested: sodium chloride, sodium citrate, sodium formate, ammonium nitrate, sodium sulfate, and disodium phosphate. These salts had no effect as demineralizers; when they were combined with an acid, no difference in the demineralization was found. When the salt neutralized the acid, the reduction in rate of demineralization was directly proportional to the reduction in the acid concentrations.

The effect of acid concentrations: Varying the molarity of hydrochloric acid solutions varied the rate of demineralization (cf. Experiment 7). It was found that the optimal concentration of hydrochloric acid for the maximal rate of demineralization was between 1 and 2 molar. An increase from 2 to 4 molar did not alter the demineralization rate and concentrations above 5 molar hydrochloric acid did not demineralize bone, they digested the bone sample.

The minimal concentration for demineralization was found to be 0.5 molar hydrochloric acid; concentrations of 0.1 and 0.05 molar were ineffective as demineralizers since they took 5 to 10 times longer than 2 molar acid.

The usual hydrochloric acid strength recommended in the literature for demineralization is a 5% solution; a 5% solution is about 0.4 molar concentration or the minimal concentration found practical in demineralization. Increase of concentration from 0.5 to 2 molar decreased the time of demineralization about 30% and did not cause any significant increase in nitrogen removal from the bone matrix.

A 2 molar concentration of trichloroacetic acid, however, was too strong for satisfactory demineralization; considerable digestion of the bone matrix occurred at this concentration (Morris and Benton[5]). Complete demineralization of these bone samples was usually indicated by a percent weight loss of about 42%, but the percent weight loss with 2 molar trichloroacetic acid exceeded 50%. A 1 molar concentration of this acid did not show an excessive percent weight loss and can be used as a demineralizer.

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SUMMARY

A. The basic limitations which govern demineralization are:

1. The surface area will determine the amount of bone demineralized per unit time because:
   a) demineralization takes place on all the bone surfaces at the same time at the same rate, and
   b) bone demineralizes only at the interface between the acid and the mineral.

2. The time necessary for bone demineralization will become progressively greater as demineralization approaches completion because of the increased distance of diffusion through the demineralized matrix.

In a practical sense, only selection of the bone weight and surface area can minimize these physical limitations. By way of example: a bone piece which measures 1 x 1 x 1 mm will demineralize in the same amount of time as a bone piece which measures 1 x 1 x 100 mm, because the time necessary for demineralization will be determined by the least dimension of the bone piece which in this case is 1 millimeter. The 100 mm surface will demineralize in exactly the same amount of time as the 1 mm of surface area since the acid acts on all surfaces at an equal rate at the same time.

The above bone samples can be demineralized with agitation in 1 molar hydrochloric acid in about 2 hours. If the bone size is increased by 1 mm on all sides so that it will be 2 x 2 x 2 mm, demineralization under the same conditions will take 8 hours or 4 times as long. The increase in the time for demineralization is due to the increased depth of matrix through which the acid and salts must diffuse. Therefore, the time for demineralization will be determined by the least dimension.

B. The optimal conditions for maximal rate of demineralization:

1. Agitation of a bone specimen will decrease the time of demineralization by 30% over a non-agitated sample of the same weight and surface area.

2. An increase in the temperature of the demineralizing solution from 25°C. (room temperature) to 36-40°C. will decrease the time of demineralization about 15%. Temperature increases above 40°C. will increase the amount of protein hydrolysis without proportionally increasing the rate of demineralization.

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3. The acid of choice for a demineralizer is hydrochloric acid because it is the most efficient, the most rapid of action, and the least harmful of all the acids tested.

4. The optimal concentration of hydrochloric acid for maximal rates of demineralization is between 1 and 2 molar. Concentrations over 2 molar do not increase the rate of demineralization; concentrations below 0.5 molar are so inefficient as to be almost valueless. The concentration of hydrochloric acid usually used in routine methods of demineralization is about 0.4 molar (5% solution of acid); by increasing this concentration from 0.4 to 1-2 molar concentration (12 to 25% solution), the time of demineralization can be decreased about 30%. 100 ml of a 1 to 2 molar concentration of hydrochloric acid will demineralize a 2 gram specimen of bone without sufficient acid depletion to warrant change of acid solution during demineralization (bone estimated at 40% mineral or 800 mg which will use 1 gram of acid or reduction of about 0.3 molar in concentration).

1041116

BIBLIOGRAPHY

1. ELLIOTT, E.: Volumetric determination of calcium in blood serum. J. Biol. Chem. 197:641-644, 1952.

2. FISKE, H., and SUBBAROW: The colorimetric determination of phosphorus. J. Biol. Chem. 66:375-400, 1925.

3. GRAY, PETER: The Microtomist's Formulary and Guide. New York, The Blakiston Company, Inc., pp 256-260, 1954.

4. KOCH, F. C., and McMEEKIN, T. L.: A new direct Nesslerization micro-Kjeldahl method and a modification of the Nessler-Folin reagent for ammonia. J. Am. Chem. Soc. 46:2066-2069, 1924.

5. MORRIS, R. E., and BENTON, R. S.: Studies in demineralization of bone with preservation of associated soft structures. Anat. Rec. 115:433-434, 1953 (Abstract).

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TABLE I

Data showing weight loss, percent weight loss, and weight of mineral removed in eight hours of demineralization in 0.5 molar hydrochloric acid for two bones of equal weight but different surface area as described in Experiment 1.

	Surface Area (sq. cm.)	Initial Weight (mg)	Weight Loss (mg)	% Difference in: Original Weight	% Difference in: Weight loss during demineralization	Mineral Removed (mg) Ca	Mineral Removed (mg) P	Mineral Removed (mg) Total Ca+P
Bar	4.64	750	224			139	55	194
Cylinder	3.41	753	181			114	44	158
Difference	1.23	3	43	nil	19%			36

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TABLE II

Data showing weight loss, percent weight loss, and weight of mineral removed in eight hours of demineralization in 0.5 molar hydrochloric acid for two bones of equal surface area but different weight as described in Experiment 2.

	Surface Area (sq. cm.)	Initial Weight (mg)	Weight Loss (mg)	% Difference in:		Mineral Removed (mg)		
				Original Weight	Weight loss During demineralization	Ca	P	Total Ca P
Bar	4.60	639	201			137	49	186
Cylinder	4.60	1129	211			142	53	195
Difference	None	490	10	57%	5%			9

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TABLE III

Data showing weight loss, weight of mineral removed, surface area, and depth of demineralization at two-hour intervals as described in Experiment 3.

Time (hours)	Wt. Loss (mg)	Wt. Mineral Removed (mg)			Demineralized volume of bone (mm3)	Surface Area of undemineralized bone (sq. cm.)	Depth of Demineralized Matrix (mm)		
		Ca	P	Total Ca+P			End	Side	Average increase per 2 hours
Before Matrix Removal:									
Start	(1131)					4.81			
2	160	106	41	147		3.74	0.54	0.50	0.52
4	227	41	16	57		3.31	0.80	0.70	0.23
6.	273	29	11	40		2.97	0.94	0.86	0.14
Total		176	68	244	192.7				
After Matrix Removal:									
Start	(445)					2.87			
2	94	49	22	71		2.00	0.54	0.50	0.52
4	127	22	8	30		1.59	0.80	0.72	0.24
6	148	16	5	21		1.35	0.98	0.86	0.16
Total		87	35	122	96.0				

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TABLE IV

Data showing weight loss, percent weight loss and weight of calcium and phosphorus removed during various time periods from bones of similar weight and surface area demineralized in 0.5 molar hydrochloric acid under conditions of agitation and lack of agitation as described in Experiment 4, parts A and B.

Time (hours)	Bone Wt. Loss		% Wt. Loss		Mineral Loss (mg)			
	Agitation Sample	Non-agitation Sample	Agitation Sample	Non-agitation Sample	Agitation Sample		Non-Agitation Sample	
					Ca	P	Ca	P
Experiment 4 A								
0	(575)	(573)						
2	83	65	14%	11%				
4	118	93	21%	16%	63	30	50	22
6	141	115	25%	20%				
8	161	131	28%	23%	18	9	17	9
Total	161	131	28%	23%	81	39	67	31
Experiment 4 B								
0	(783)	(788)						
24	287	243	37%	31%	129	75	103	64
50	341	322	44%	41%	20	15		
69	---	342	---	44%	---	--	34	24
Total	341	342	44%	44%	149	90	137	88

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TABLE V

Data showing weight loss, percent weight loss, and weight of calcium and phosphorus removed from four bones of similar weight and surface area demineralized in 0.5 molar hydrochloric acid at various temperatures for eight hours as described in Experiment 5.

Solution Temperature	Initial Bone Weight (mg)	Bone Wt. Loss (mg)	% Wt. Loss	Mineral Removed (mg)			
				Ca	P		Total Ca+P
26°C.	586	128	22%	69	35	=	104
36°C.	587	146	25%	76	38	=	114
46°C.	585	161	27%	80	38	=	118
56°C.	587	189	32%	85	41	=	126

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TABLE VI

Data showing percent weight loss, the grams of acid and millimoles of hydrogen ion used during demineralization to remove grams and/or millimoles of calcium and phosphorus, and the ratio of hydrogen ion to mineral removal during eight hours for a series of acids at 1 molar concentration as described in Experiment 6.

Acid	% Wt. Loss	Grams of acid and % of a 1 molar solution used during demineralization		Millimoles of H+ used during demineralization	Ca and P Removed (mg)				Millimoles of Ca+P removed during demineralization	Ratio of Millimoles Hydrogen to remove 1 millimole of Ca+P
		Grams	% of 1 molar solution		Ca	P	=	Total Ca+P		
Hydrochloric	31%	0.1156	3%	3.17	88	32	=	120	3.233	0.98 : 1
Trichloroacetic	16%	0.3153	2%	1.93	42	20	=	62	1.709	1.12 : 1
Formic	7%	0.0492	1%	1.07	22	9	=	31	0.855	1.20 : 1
Phosphoric	15%	0.3951	4%	2.01	40	3	=	43	1.105	1.81 : 1
Nitric	30%	0.6131	10%	9.73	75	30	=	105	2.869	3.39 : 1
Hydrobromic	28%	1.5763	20%	19.48	69	30	=	99	2.697	7.22 : 1
Acetic	1%	0.2311	4%	3.85	8	3	=	11	0.321	11.99 : 1
Lactic	4%	1.0170	11%	11.29	16	3	=	19	0.528	21.38 : 1
Citric	3%	6.4513	34%	33.58	nil	nil	=	nil	nil	

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TABLE VII

Data showing weight loss, percent weight loss, and milligrams of calcium and phosphorus removed from bone samples of similar weights and surface areas when demineralized in different concentrations of hydrochloric acid for eight hours as described in Experiment 7.

Concentration of hydrochloric acid	Initial Bone Weight (mg)	Weight Loss (mg)	Percent Weight Loss	Mineral Removed (mg) Ca	P		Total Ca+P
4 molar	637	227	35%	130	51	=	181
2 molar	637	226	35%	129	52	=	181
1 molar	635	200	32%	118	48	=	129
0.5 molar	633	164	26%	87	42	=	129
0.1 molar	629	62	10%	53	21	=	64
0.05 molar	633	29	5%	37	14	=	51

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[redacted]

Figure 2.

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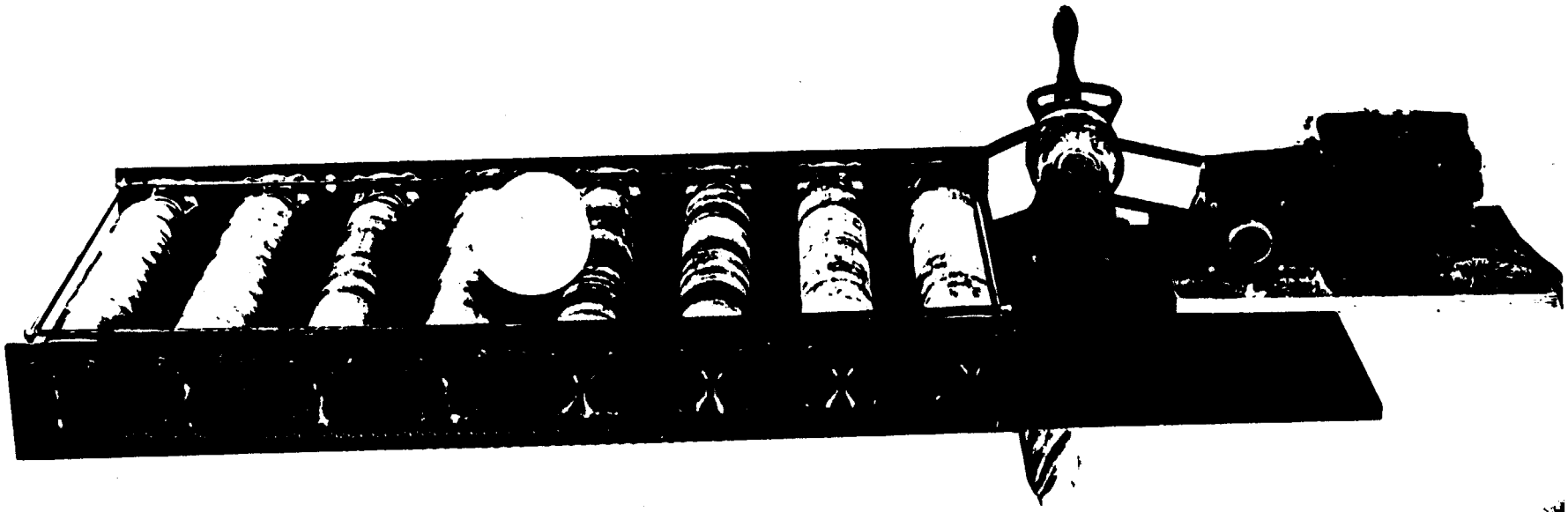

Figure 1.

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