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STUDIES ON DEMINERALIZATION OF BONE

I. THE BASIC FACTORS OF DEMINERALIZATION

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More than 40 papers in scientific journals during the last 10 years report presumably improved methods for the demineralization of bone, each of which is said to be superior to the previous methods. One of 2 conclusions may be drawn from the relatively large number of papers that report 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. Everyone who has worked in this field agrees that all methods of demineralization are not equally good; therefore, the difficulty must lie in the lack of comparative standards for evaluation.

A review of many experimental methods used by a number of technical innovators reveals this lack of standards. Inasmuch as the repeatability of a technic 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 fail to give adequate information for repetition of the method or evaluation of the 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. Inasmuch as the results are altered by the type of bone used, lack of exact definition of the sample of bone 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 not defined. The temperature, frequency of changing the solution, 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 with a needle and estimate from the resistance whether or not demineralization was complete.

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

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

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

The scope and purpose of this present work, therefore, is 3-fold: (1) to define standard conditions for the comparison of the efficiency of methods of demineralization; (2) to determine the basic factors that are the limitations of any method of demineralization; and (3) to determine whether or not the time required for demineralization can be shortened by varying the experimental conditions of temperature, agitation, type of acid, and molarity of the acid bath.

MATERIALS AND METHODS

Standard bone. For all of the experiments, sections of dense cortical bone from the shaft of an air-dried, adult human femur were used. This bone had been previously analyzed 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 per cent. The average composition of the bone was: nitrogen, 5 per cent; calcium, 21 per cent; phosphorus, 10 per cent; fat, 11 per cent; carbon dioxide, 3 per cent; and water removed by heating at 125 C. for 4 weeks, 18 per cent. The amount of calcium and phosphorus for the standard bone was 0.81 mg. per cu. mm. of bone.

Method of preparing bone samples. Samples of bone 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 fragments of metal from contaminating the sample of bone) and deep grain (which removed the bone dust without its being ground into the minute canals within the specimen).

The pieces of bone 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.

Experimental conditions. In all experiments, the following conditions were adhered to unless otherwise specified:

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 samples of bone 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 change of solution in order to prevent contamination or loss of the solution through transfer.

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

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FIG. 1. The roller agitator machine provides constant motion of the sample of bone during demineralization; a polyethylene container (with the demineralizing solution and sample of bone) is shown in place. The speed of the machine may be varied from 60 to 120 r.p.m.

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, and the weighings were made by the other investigator in order to keep errors constant. Repeated drying and weighings indicate that this error was not more than ±1 mg.

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

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

Chemical technics. Four quantitative chemical determinations were made on each demineralizing solution. The individual determinations were made in quadruplicate.

Acid standardization: The solutions of acid were titrated before and after demineralization with a standard solution of sodium hydroxide, using bromthymol blue, phenolphthalein, or bromo cresol green as the indicator.

Nitrogen determinations: The total content of nitrogen was determined by digestion with sulfuric acid and steam distillation of the digestion mixture, as described by Koch and McMeekin.[4] Boric acid was used for collecting the distillate, which was Nesslerized[1] and read at 10 minutes in a photoelectric colorimeter at 425 mμ.

Phosphorus determinations: Phosphorus ion was determined by the method of Fiske and Subbarrow.[2] The technic was modified by increasing the amount of molybdate reagent 10-fold (in place of the distilled water) when making determinations on chelating acids, owing to the binding of the molybdate reagent by these compounds.

Calcium determinations: Calcium was determined by titration of the calcium ion with disodium versenate, using purpurate as the indicator.[1] The technic was modified by precipitation of the calcium ion with ammonia water and ammonium oxalate, and isolation by centrifugation before titration, in order to eliminate interfering substances.

EXPERIMENTS

Section I: 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

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TABLE 1

LOSS IN WEIGHT, PER CENT OF WEIGHT LOST, AND AMOUNT OF MINERAL REMOVED IN 8 HOURS OF DEMINERALIZATION IN 0.5 M HYDROCHLORIC ACID FOR PIECES OF BONE WITH DIFFERENT SURFACE AREAS OR DIFFERENT WEIGHTS

(Experiments 1 and 2)

	Surface Area	Initial Weight	Weight Loss	Per Cent Difference in		Mineral Removed		
				Original weight	Weight loss during demineralization	Ca	P	Total
	sq. cm.	*mg.*	*mg.*			*mg.*	*mg.*	*mg.*
Bones of equal weight:								
Bar	4.64	750	224			139	55	194
Cylinder	3.41	753	181			114	44	158
Difference	1.23	3	43	nil	19			36
Bones of equal surface area:								
Bar	4.60	639	201			137	49	186
Cylinder	4.60	1129	211			142	53	195
Difference	None	490	10	57	5			9

to form a bar and a cylinder having the same weight, but different surface areas: bar of bone—750 mg., with 4.64 sq. cm. of surface area; cylinder of bone—753 mg., with 3.41 sq. cm. of surface area; proportion of the surface area of the smaller to the larger bone, 73 per cent.

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

Results. The surface area of the smaller bone was 73 per cent 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 1). 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: bar of bone—639 mg., with 4.60 sq. cm. of surface area; cylinder of bone—1129 mg., with 4.60 sq. cm. of surface area; proportion of the weight of the smaller to the larger bone, 57 per cent.

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

Results. The surface areas of the 2 samples of bone were practically identical, but the smaller bone was 57 per cent of the larger bone by weight. After 8 hours of demineralization, the difference in the weight lost by the 2 samples was only 10 mg., and a difference of only 9 mg. of calcium and phosphorus removed

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(Table 1). Thus, bones with the same surface area, but different weights, had almost no difference in loss of weight or in the amounts of mineral removed.

Experiment 3: The characteristics of mineral removal and diffusion. A piece of standard bone was shaped as a bar and demineralized in 0.5 molar hydrochloric acid for 6 hours, after which the experiment was stopped, and the demineralized matrix was removed. The surface area and weight were again determined: original specimen—1131 mg., with 4.81 sq. cm. of surface area; after removal of the matrix—445 mg., with 2.87 sq. cm. of surface area.

The bone was further demineralized under the same conditions for a second 6-hour period. In both parts of the experiment, the bone was weighed and x-ray films were made (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 sample of bone 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 revealed that the process was essentially the same on all external surfaces of the bone (Table 2). Because of this, the form of the undemineralized portion was not changed (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 at the ends of the sample of bone, and it was 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.

TABLE 2

LOSS IN WEIGHT, AMOUNT OF MINERAL REMOVED, SURFACE AREA, AND DEPTH OF DEMINERALIZATION AT 2-HOUR INTERVALS

(Experiment 3)

Time	Weight and Loss in Weight	Amount of Mineral Removed			Demineralized Volume of Bone	Surface Area of Undemineralized Bone	Depth of Demineralized Matrix		
		Ca	P	Total			End	Side	Average increase per 2 hours
Before Removal of Matrix									
hr.	*mg.*	*mg.*	*mg.*	*mg.*	*cu. mm.*	*sq. cm.*	*mm.*	*mm.*	*mm.*
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 Removal of Matrix									
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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FIG. 2. Contact print of x-ray negative of a piece of demineralizing bone (Experiment 3). The demineralized area of bone is represented by the gray border about the central black core of undemineralized bone. The demineralized portions are equal on all sides, evidence of the simultaneous action of acid on all surfaces.

The more deeply the demineralizing agent penetrated into the matrix of the bone sample, the more slowly it penetrated per unit time (Table 2). The depth of demineralization at the end of the sample during the first 2-hour period was 0.54 mm.; during the second 2-hour period, the increase in depth was 0.26 mm.; and during the third 2-hour period it was 0.14 mm. Thus the increase in depth of demineralization for any period of time was approximately 50 per cent of that for the previous 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 2).

This decrease in the depth of bone demineralized each 2-hour period was related neither to surface area nor to the weight of the piece of bone. Rather, it was related to the increasing thickness of demineralized matrix between the solution and the undemineralized bone. This was demonstrated by comparing the increase in the depth of demineralized matrix during the last 2 hours before, and the first 2 hours after removal of the matrix. These increases in depth were 0.14 mm. and 0.54 mm. The only change of conditions between the 2 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 seemed to be the result of the process of diffusion through the matrix.

The weight of calcium and phosphorus removed per 2-hour period also decreased. During the first interval, 147 mg. of calcium and phosphorus were removed; during the second interval, 57 mg.; and during the third 2-hour interval, 40 mg. In the second 6-hour period, there were decreases in removal of mineral that were proportional to those of the first period. Less mineral was removed in the second 6-hour period, and this reflected the difference in surface area, or the amount of mineral available to the action of acid (see Experiments 1 and 2).

Measurements from the surface of the piece of bone to the sharp boundary of undemineralized bone in x-ray negatives (Fig. 2) provided the data for

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calculating the volume of bone that was demineralized, inasmuch as the amount of mineral removed was determined chemically. During the first 6-hour period, 0.790 mg. of calcium and phosphorus were removed per cu. mm. of demineralized bone; during the second 6-hour period, 0.787 mg. were removed per cu. mm. (Table 2). The analyzed standard bone (*cf.*, Materials and Methods) had an average concentration of calcium and phosphorus of 0.81 mg. per cu. mm.

DISCUSSION OF THE BASIC FACTORS OF DEMINERALIZATION

The basic factors that constitute the limitations of any method of demineralization of bone were demonstrated in the 3 experiments described.

1. The greater the surface area, the greater the volume of bone that was demineralized per unit time. Data from Experiment 2 revealed that when the surface areas of pieces of bone 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 2 bones are equal, the amount of demineralization will be equal, regardless of the weights of the bone; conversely, if the surface areas are unequal, the amount of demineralization will vary with the difference in surface areas.

2. Demineralization proceeded simultaneously on all surfaces of the bone at the same rate, regardless of its surface area.

3. Demineralization occurred only at the interface between the demineralizing solution and the bony mineral. The amount of mineral removed in the experimental sample was approximately 3 per cent (Experiment 3) less than the amount of mineral in the same volume of the analyzed standard bone. The close similarity between the analyzed control standard and the experimental bone, as well as the sharp boundary of undemineralized bone in the x-ray negatives, strongly indicated that there was little or no demineralization except at the surface layer of the undemineralized part of the bone.

4. As demineralization continued, the process became progressively slower owing to the increased distance of diffusion through demineralized matrix. The results of Experiment 3 indicated that the rate of demineralization decreased as the thickness of demineralized matrix increased; 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. The demineralizing acid must diffuse through the layer of matrix in order to reach the surface of the undemineralized bone, and the minerals of the bone must diffuse outward through the same material. The greater the distance of diffusion, the longer the time required for these substances to diffuse. Therefore, the progressive slowing of the demineralization process is a reflection of the increased time required 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

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continuously decreased, thus resulting in lesser amounts of bone minerals being exposed to acid at the mineral-acid interface. This was demonstrated in Experiment 3, in which 244 mg. of mineral were removed in 6 hours from a piece of bone with a surface area 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 yielded 122 mg. of mineral when the surface area was reduced from 2.87 to 1.35 sq. cm. Thus, when the surface area was reduced by approximately 50 per cent, the amount of mineral removed was reduced by approximately 50 per cent. In both instances, the depth of demineralized matrix was the same, indicating that the rate of demineralization was the same.

It must be expected, therefore, that a smaller *volume* of bone will be demineralized as the process approaches completion, owing to the continued reduction in surface area. However, the *greater time* required for demineralization is not the result of reduction in surface area, but is a reflection of the increasing distance of diffusion; the rate per unit of surface area remains constant.

Section 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 2 groups of conditions that affect demineralization, in order to obtain the greatest efficiency within these limitations: (1) factors that increase the rate of diffusion (*e.g.*, temperature and agitation); (2) factors that increase the efficiency of the demineralizer (*e.g.*, type and concentration).

Experiment 4: The effect of agitation on the rate of demineralization. In Experiment 4A, 2 pieces of standard bone were shaped to approximately the same weight and surface area: one piece (to be agitated) weighed 575 mg., and had a surface area of 3.36 sq. cm.; the other (not to be agitated) weighed 573 mg., and had a surface area of 3.36 sq. cm. For Experiment 4B, 2 more pieces of standard bone were cut: one piece (to be agitated) weighed 783 mg., and had a surface area of 3.92 sq. cm.; the other (not to be agitated) weighed 788 mg., and had a surface area of 3.93 sq. cm.

The conditions were the same except for the length of time the experiment was conducted; *i.e.*, Experiment 4A was concluded after 8 hours of demineralization, whereas Experiment 4B was continued for 69 hours of demineralization. In both experiments, the samples were placed in 0.5 molar hydrochloric acid, and the containers with the samples for agitation were placed upon a roller-agitator revolving at 85 r.p.m. The non-agitated samples were left undisturbed in their containers between weighings. The samples were cleared in beechwood creosote, and the depths to which the mineral was removed from the samples were measured. The times of weighing the samples and changing the solution are recorded in Table 3.

Results. In Experiments 4A and 4B, the surfaces of the non-agitated samples rapidly collected a layer of bubbles that eventually covered the samples in an envelope of gas. This gaseous 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

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TABLE 3

LOSS IN WEIGHT, PER CENT OF WEIGHT LOST, AND AMOUNT OF CALCIUM AND PHOSPHORUS REMOVED DURING VARIOUS TIME PERIODS FROM BONES OF SIMILAR WEIGHT AND SURFACE AREA DEMINERALIZED IN 0.5 M HYDROCHLORIC ACID UNDER CONDITIONS OF AGITATION AND LACK OF AGITATION

(Experiment 4, parts A and B)

Time	Loss of Weight		Per Cent of Weight Lost		Loss of Mineral			
	Agitation sample	Non-agitation sample	Agitation sample	Non-agitation sample	Agitation sample		Non-agitation sample	
					Ca	P	Ca	P
			Experiment 4A					
hr.	*mg.*	*mg.*			*mg.*	*mg.*	*mg.*	*mg.*
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 4B					
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

not constant, inasmuch as it was necessary to remove the gaseous envelope when the samples were dried for the periodic weighings. This led to a slight increase of the rate of demineralization for the non-agitated specimens.

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

Further data on the difference between agitated and non-agitated samples are listed in Table 3. In Experiment 4B (69 hours of demineralization), the agitated specimen lost 44 per cent of its weight in 50 hours and was completely demineralized, whereas the non-agitated specimen did not attain this per cent of loss of weight until 69 hours of demineralization. The agitated specimen was demineralized at least 38 per cent faster than the non-agitated specimen. Similarly, in Experiment 4A (8 hours of demineralization), the agitated specimen had 21 per cent loss of weight in 4 hours, but the non-agitated specimen did not have this per cent of loss of weight until 6 hours of demineralization. Thus, the agitated bone demineralized to the same extent in approximately 33 per cent less time than the non-agitated sample.

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TABLE 4

LOSS IN WEIGHT, PER CENT OF WEIGHT LOST, AND AMOUNT OF CALCIUM AND PHOSPHORUS REMOVED FROM 4 BONES OF SIMILAR WEIGHT AND SURFACE AREA DEMINERALIZED IN 0.5 M HYDROCHLORIC ACID AT VARIOUS TEMPERATURES FOR 8 HOURS

(Experiment 5)

Temperature of Solution	Initial Weight of Bone	Loss of Weight	Per Cent of Weight Lost	Mineral Removed		
				Ca	P	Total
	mg.	*mg.*		*mg.*	*mg.*	*mg.*
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

Experiment 5: The effect of variation in temperature on the rate of demineralization. Four samples of approximately equal weight (586, 587, 585, and 587 mg., respectively) and surface area (2.80 sq. cm.) were prepared from the same part of the standard bone. Each of the samples was placed in a separate portion of 0.5 M solution of hydrochloric acid in order to be demineralized at a different temperature, *i.e.*, 26, 36, 46, and 56 C., ±1 C. The bone was weighed every hour during the 8-hour period of demineralization, and the acid solutions were changed every 2 hours.

Results. The data (Table 4) indicate that raising the temperature of the demineralizing solution increased the amount of mineral removed per unit time, but above 36 C. undesirable side effects occurred. These are more evident if the amount of mineral removed is expressed as a percentage of the total loss of weight at each temperature, *i.e.*, the amount of mineral that was removed accounted for 81 per cent of the loss of weight at 26 C., for 78 per cent at 36 C., for 73 per cent at 46 C., and for 66 per cent at 56 C. Although more mineral was removed at the higher temperatures, larger amounts of other materials were also removed (as much as 33 per cent at 56 C.). Chemical studies indicated that these materials were chiefly proteins and fats of the organic matrix.

Similarly, there was a decrease in the efficiency of demineralization as the temperature was raised, *i.e.*, increases in temperature from 26 to 36 C., to 46 C., and to 56 C. resulted, respectively, in 10 per cent, 4 per cent, and 6 per cent increase in the amount of mineral removed. Thus, the rate of removal of mineral was not proportional to the increase in temperature. The most advantageous temperature seemed to be approximately 36 C., inasmuch as there was maximal removal of mineral with minimal, undesirable side effects.

Experiment 6: The efficiency of various acids in demineralization. Sections of standard bone were prepared with similar weights (400 to 434 mg.) and surface areas (0.218 to 0.235 sq. cm.). These samples were placed in 1 M concentrations of hydrochloric, nitric, hydrobromic, phosphoric, trichloracetic, formic, lactic, acetic or citric acid, demineralized for 8 hours, and weighed every hour. Acid solutions were changed every 2 hours. At the end of the experiment, the samples

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TABLE 5

PER CENT OF WEIGHT LOST, GRAMS OF ACID AND MILLIMOLES OF HYDROGEN ION USED DURING DEMINERALIZATION, AND THE RATIO OF HYDROGEN ION TO MINERAL REMOVED DURING 8 HOURS, FOR A SERIES OF ACIDS AT 1 M CONCENTRATION

(Experiment 6)

Acid	Per Cent of Weight Lost	Grams of Acid and Per Cent of 1 M Solution Used During Demineralization		Millimoles of H^+ Used During Demineralization	Ca and P Removed			Millimoles of Ca + P Removed During Demineralization	Ratio of Millimoles of Hydrogen to Remove 1 Millimole of Ca + P
		Grams	Per Cent of 1 M Solution		Ca	P	Total		
					mg.	*mg.*	*mg.*		
Hydrochloric	31	0.1156	3	3.17	88	32	120	3.233	0.98:1
Trichloracetic	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	

were cleared in beechwood creosote, and the depth of the demineralized matrix was measured.

Results. The relative rates of breakdown and removal of mineral by the acids tested are summarized in Table 5, based on the per cent of loss in weight of the bones after 8 hours of demineralization. The total mineral removed by each acid is also summarized in Table 5.

When the per cent of loss in weight, depth of demineralized matrix, and amount of 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 classed into 3 groups, based on separations that occurred in the data:

	Per Cent of Loss in Weight of Bone	Milligrams of Mineral Removed	Solubility of Calcium Salts of the Acid in Gm. per 100 ml. of Water at 25 C.*	Range of Dissociation Constants of the Acids*
1.	28 to 31	99 to 120	125 to 315	7 to 9×10^{-1}
2.	7 to 16	31 to 62	35 to 70	2×10^{-1} to 1.76×10^{-3}
3.	1 to 4	0 to 19	0.25 to 17	1.38×10^{-3} to 1.76×10^{-5}

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

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 1), and the lowest rates of demineralization were observed with acids

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that had the lowest solubility of calcium salts and dissociation constants (Group 3).

A more detailed summary of the grouping of the various acids is listed below, where the acid with the highest value in a category is named at the top of that column, and the acid with the lowest value is at the bottom:

Per Cent of Loss of Weight	Depth of Demineralized Matrix	Mineral Removed	Solubility of the Calcium Salts	Dissociation Constant
1. Hydrochloric	Hydrochloric	Hydrochloric	Nitric	Hydrobromic
Nitric	Hydrobromic	Nitric	Hydrochloric	Nitric
Hydrobromic	Nitric	Hydrobromic	Hydrobromic	Hydrochloric
2. Trichloracetic	Phosphoric	Trichloracetic	Trichloracetic	Trichloracetic
Phosphoric	Trichloracetic	Phosphoric	Acetic	Phosphoric
Formic	Formic	Formic	Phosphoric	Formic
3. 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 of the bone is determined by the dissociation of the acid, and the solubility of the calcium salts partially regulates the rate of removal by diffusion through the demineralized matrix. It is difficult to separate these 2 components experimentally because the dissociation constants of acids usually parallel the solubilities of their calcium salts. Acetic acid, however, did not follow this general rule, and the solubility of calcium acetate stands higher in the classification than does the dissociation of acetic acid. 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, acetic acid should rank in the group with trichloracetic acid, and not as the poorest demineralizing agent tested. From this, it would seem that the solubility of the calcium salt of an acid is of no benefit if the mineral is not broken down in bone. Thus, it is reasonable to conclude that the rate of demineralization is chiefly determined by the rate of breakdown of mineral, which is related to the dissociation constant of the acid.

Titrations of the concentrations of acid at the end of the experiment revealed the amount of acid that was utilized in the process of demineralization. The maximal amount of acid utilized was 34 per cent in the case of citric acid (Table 5); the other acids averaged approximately 10 per cent utilization during the 8 hours of demineralization. Inasmuch as a 10 per cent reduction in molarity only reduced the concentration from 1 to 0.9 M, an adequate amount of acid was present for demineralization (*cf.*, Experiment 7 for the minimal concentration of acid for effective demineralization).

A measure of the efficiency of an acid in the removal of mineral from bone was established by determining the number of millimoles of hydrogen used to remove 1 millimole of the mixture of calcium and phosphorus in bone. This

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ratio of hydrogen to mineral removed is recorded in Table 5. The data revealed that hydrochloric acid was the most efficient for removal of mineral, inasmuch as 0.98 millimole of hydrogen removed 1 millimole of the mixture of minerals. Hydrochloric acid was followed by trichloracetic, formic, and phosphoric acids, all of which had a ratio of less than 2:1. All the other acids had a ratio of more than 3:1, and this was regarded as inefficient. Chemical determinations revealed that the acids with a ratio higher than 3:1 had a higher concentration of nitrogen in the solution following demineralization. It is suspected that the excessive amount of hydrogen was used in hydrolyzing protein rather than removing mineral. This same phenomenon of high removal of nitrogen from the demineralizing bone was also noticed in the higher concentrations of trichloracetic acid.

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

Experiment 7: The effect of concentrations of an acid upon the rate of demineralization. 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, 2, 1, 0.5, 0.1, and 0.05 M. The samples were weighed every hour, and the acid solutions were changed every 2 hours. At the end of the experiment the pieces of bone 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 the per cent of loss in weight or in the amount of mineral removed (Table 6) with 4 and 2 M hydrochloric acid. The loss in weight of the sample of bone in 1 M acid was 89 per cent of the loss in weight of the bone in 2 M hydrochloric

TABLE 6

LOSS IN WEIGHT, PER CENT OF WEIGHT LOST, AND MILLIGRAMS OF CALCIUM AND PHOSPHORUS REMOVED FROM BONE SAMPLES OF SIMILAR WEIGHTS AND SURFACE AREAS DEMINERALIZED IN DIFFERENT CONCENTRATIONS OF HYDROCHLORIC ACID FOR 8 HOURS

(Experiment 7)

Concentration of Hydrochloric Acid	Initial Weight of Bone	Loss in Weight	Per Cent of Weight Lost	Mineral Removed		
				Ca	P	Total
M	*mg.*	*mg.*		*mg.*	*mg.*	*mg.*
4	637	227	35	130	51	181
2	637	226	35	129	52	181
1	635	200	32	118	48	166
0.5	633	164	26	87	42	129
0.1	629	62	10	53	21	64
0.05	633	29	5	37	14	51

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acid, and the mineral removed in 1 M acid was 92 per cent of that removed in 2 M acid. The removal of mineral and loss in weight with 0.5 M hydrochloric acid was 71 per cent of that in 2 M acid. Demineralization in 0.1 and 0.05 M concentrations was of such low order that these concentrations were regarded as ineffective. A peculiarity was noticed in demineralization with 0.1 and 0.05 M hydrochloric acid. In these, the amount of mineral removed exceeded the amount of weight that was lost (Table 6). A subsequent experiment indicated that this was the result of absorption of water by the demineralized matrix of the bone, inasmuch as the differences in loss of weight in the completely demineralized bones disappeared when the samples were dried at 125 C. for 6 days.

It was evident that increasing the molarity of the acid beyond 2 M did not result in an increase in the efficiency of demineralization, and the process was unduly prolonged when the concentration was less than 0.5 M. The optimal concentration for hydrochloric acid as a demineralizing agent was between 1 and 2 M.

DISCUSSION OF THE EFFECTS OF ALTERING THE CONDITIONS OF DEMINERALIZATION

In Section I of this paper, the inescapable limitations of any process of demineralization are described. The experiments described in Section II were devoted to determining the optimal conditions for demineralization of bone, and 4 factors were studied: the effect of agitation, the effect of temperature, the relative value of several acids as demineralizing agents, and the optimal concentrations of an acid for maximal efficiency. Using the optimal conditions for each of these factors made it possible to reduce the time of demineralization more than 50 per cent, as compared with the usual methods and the same reagents.

Effect of agitation. Continuous agitation of the pieces of bone increased the amount of demineralization approximately 30 per cent. The rate of agitation is not important as long as the bone is continually tumbling in the solution. The rate of 85 r.p.m. was used in these studies because it resulted in continuous motion of the bone, regardless of the size of the pieces.

There are probably 3 reasons for the shortening of the time of demineralization by agitation. Agitation prevents the accumulation of bubbles of gas on the surface, which would limit the contact with the demineralizing acid and reduce the rate of demineralization. Secondly, agitation prevents the piece of bone from resting on the bottom of the container, which would reduce its area of contact with the demineralizing acid. Thirdly, agitation assures an equal distribution of bone salts and acid in the demineralizing solution. The removal of mineral from bone is chiefly a process of diffusion, 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 temperature. An increase in the temperature of the demineralizing solution from 25 C. (average room temperature) to 36 to 40 C. decreased the time of demineralization approximately 15 per cent. Increases of temperature above 40 C. resulted in a continued decrease in the time for demineralization, but the amount of mineral removed was not proportional to this decrease; in

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Clear in 1:1 mixture of oil of wintergreen and absolute ethanol, then in 1:1 mixture of oil of wintergreen and xylene (approximately 12 hours).

6. Embed in paraffin (melting point, 52 C.) *in vacuo* and block; cut sections 4 microns in thickness, mount, and hydrate (approximately 2 hours).

7. Immerse sections in 5 per cent aqueous solution of ammonium chloride (approximately 30 minutes).

8. Apply Wright's stain, dehydrate, and clear in butyl alcohol and xylene; put coverslip in position with Clarite (approximately 15 minutes). If a Giemsa preparation is desired, apply the stain after step 7, differentiate, and coverslip after clearing in xylene (approximately $2^1/_2$ hours).

Results. There is practically no shrinkage of the tissue (Fig. 1), and the cytologic details are well preserved and vividly stained (Fig. 2). The total time required for preparation of slides stained with Wright's stain is approximately 26 hours, and for those stained with Giemsa's stain, approximately 28 hours.

DISCUSSION

The experiments on staining of bone and associated soft tissues (after demineralization of the bone) indicated that the critical element in the various procedures was the fixing of the tissues, *i.e.*, cytology was not altered by the acid, during demineralization, if the tissues were fixed well. A fixative that provides and maintains good preservation during demineralization should have the following characteristics:

1. The fixative should be relatively rapid in action owing to the fact that the cells in bone marrow undergo irreversible changes shortly after death or after removal of a piece of bone during surgery. The density of the bone and the limited amount of exposed surface make it desirable that the fixative penetrate well, in order that the deeper portions of the tissue may be preserved prior to disintegration of the cells.

2. The fixative should not harden the tissue excessively, inasmuch as further hardening that results from treatment in acid and embedding in paraffin may make it practically impossible to cut sections in which the histologic pattern is preserved.

3. The fixative should bring about precipitation of protein in an insoluble form. If the precipitate is not insoluble, treatment with acid during demineralization will result in excessive hydrolysis.

The advantages and disadvantages of various fixing solutions were observed in several experiments. Although Orth's, Zenker's, and Helly's fluids usually provide excellent preservation of cytologic structures, they were unsatisfactory for preserving bone marrow during demineralization. These fixatives were slowly penetrant and fixed only the outer millimeter or so of tissue; although this region had excellent preservation of cellular elements, the bone could not be used for study inasmuch as it was fragmented during the sawing of the specimen. In addition, dichromate fixatives made the tissue extremely hard, and after demineralization the sections tended to shatter and tear.

Metallic substances such as iodine (in methanol) and osmic acid provided

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A 2 M concentration of trichloracetic acid, however, was too strong for satisfactory demineralization, inasmuch as considerable digestion of the matrix occurred at this concentration.[5] Complete demineralization of these samples of bone was usually indicated by a loss in weight of approximately 44 per cent, but the loss with 2 M trichloracetic acid exceeded 50 per cent. A 1 M concentration of this acid did not lead to an excessive loss in weight, and it may be used as a demineralizing agent.

SUMMARY

1. The surface area determines the amount of bone that is demineralized per unit time, inasmuch as the process is active on all the surfaces at the same time and same rate, and bone demineralizes only at the interface between the acid and the mineral. Thus, a piece of bone 1 x 1 x 100 mm. will demineralize in the same time that is required for a piece 1 x 1 x 1 mm.

2. The time required for demineralization of bone becomes progressively greater as the process approaches completion, owing to the increased distance for diffusion through the demineralized matrix. For example, a piece of bone 1 x 1 x 1 mm. can be demineralized with agitation in 1 M hydrochloric acid in approximately 2 hours, but approximately 8 hours (under the same conditions) are required if the piece of bone is 2 x 2 x 2 mm.

3. Approximately 30 per cent less time is required for demineralization of a piece of bone that is agitated during the time in acid, as compared with the time required for a piece (with the same weight and surface area) that is not agitated.

4. Increasing the temperature from 25 C. (room temperature) to 36 to 40 C. results in approximately a 15 per cent reduction of the time required for demineralization. Temperatures above 40 C. lead to increased hydrolysis of protein without proportionally increasing the rate of demineralization.

5. Hydrochloric acid is the best one to use as a demineralizing agent. It is the most efficient, most rapid in action, and the least harmful of the several acids tested.

6. The optimal concentration of hydrochloric acid for maximal rates of demineralization is between 1 and 2 M. Concentrations more than 2 M do not increase the rate of demineralization, and those less than 0.5 M are inefficient. One hundred milliliters of a 1 to 2 M solution demineralizes a 2 Gm. specimen of bone with so little depletion of acid that a change of the acid solution during the process is hardly warranted.

SUMMARIO IN INTERLINGUA

1. Le area del superficie determina le quantitate de osso que es dismineralisate in un certe spatio de tempore, nam le processo es active simultaneemente e con le mesme grado de intensitate a omne le superficies, e le osso es dismineralisate solmente al interficie de acido e mineral. Assi un pecia de osso de 1 per 1 per 100 mm es dismineralisate in le curso del mesme tempore que es requirite pro le dismineralisation de un pecia de osso de 1 per 1 per 1 mm.

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2. Le tempore requirite pro le dismineralisation de osso cresce progressivemente in tanto que le processo approcha su completion. Isto es explicate per le crescente distantia del diffusion a transverso le jam dismineralisate portion del matrice. Per exemplo, un pecia de osso de 1 per 1 per 1 mm pote esser dismineralisate con agitation in acido hydrochloric de un concentration de 1 M in le curso de circa 2 horas, sed sub le mesme conditiones circa 8 horas es requirite si le dimensiones del pecia de osso es 2 per 2 per 2 mm.

3. Le tempore requirite pro le dismineralisation de un pecia de osso es reducite per circa 30 pro cento si illo es agitate durante le tempore de immersion in le acido. Iste reduction es constatate per comparation con le tempore requirite pro le dismineralisation de un pecia de osso del mesme peso e del mesme area superficial que non es agitate durante su immersion in le acido.

4. Un augmento del temperatura ab 25 C (temperatura normal de interior) a 36 o 40 C resulta in un reduction de circa 15 pro cento in le tempore requirite pro le dismineralisation. Temperaturas de plus que 40 C resulta in un augmento del hydrolyse de proteina sin acceleration proportional del processo dismineralisante.

5. Acido hydrochloric es le melior agente de dismineralisation. Illo es le plus efficace, ha le plus rapide action, e es le minus nocive inter le varie acidos probate.

6. Le concentration optimal de acido hydrochloric pro grados maximal de dismineralisation es inter 1 e 2 M. Concentrationes de plus que 2 M non accelera le processo dismineralisante, e concentrationes de minus que 0,5 M es inefficace. Cento ml de un solution de 1 a 2 M dismineralisa un specimen de 2 g de osso con si basse grados de depletion del acido que nulle renovation del solution es indicate in le curso del processo.

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STUDIES ON DEMINERALIZATION OF BONE

II. THE EFFECT OF ELECTROLYTIC TECHNICS IN DEMINERALIZATION

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Richman, Gelfand, and Hill[3] in 1947 introduced the electrolytic method for demineralization of bone, and this technic has received widespread acceptance because it is said to demineralize bone more rapidly than any other method. Lillie and his associates,[1] and Rosen and Friedland[4] presented evidence that the decreased time of demineralization by the electrolytic method was the result of an increase in the temperature of the solution.

The present experiments were designed to determine if electrolysis does shorten the time of demineralization, under constant conditions for all known variables except the electric current.

MATERIALS AND METHODS

1. An electrolytic machine was constructed which controlled the electric current, the temperature of the demineralizing solution, and the rate of agitation. The apparatus was constructed in 3 parts, *i.e.*, a rotating electrode carrier and a container for the solution, a source of direct current, and a constant temperature bath (Fig. 1).

The electrode carrier was constructed of 2 lucite discs that were connected by an aluminum shaft and bearing. The lower disc rotated in the bearing mounted in the upper disc; electrical contact was maintained during rotation by a commutator of copper rings and silver brushes. A thermometer, a mercury thermostat, and 2 platinum electrodes were mounted on the lower disc of the carrier. The electrodes were made of 16-gauge platinum wire wound to a 1-cm. diameter, and were placed 1 cm. apart. The amount of platinum wire submerged in the solution was the same for both electrodes.

The source of direct current was a Heath powerpack that provided controlled wattage from 0 to 12 volts at 0 to 8 amperes.

The temperature of the electrolytic solution was controlled by circulating water through a coil of 5-mm. polyethylene tubing in the container. The polyethylene coil was connected to a constant temperature bath through a pump that was controlled by the thermostat mounted in the electrode carrier. The

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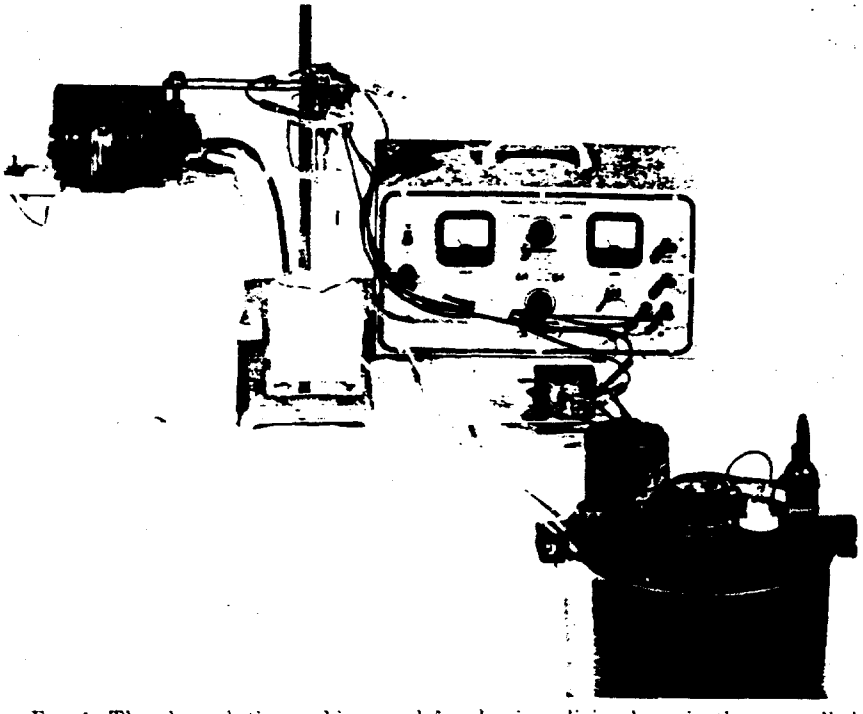

FIG. 1. The electrolytic machine used for demineralizing bone in the controlled experiments consisted of: a geared motor driving the electrode carrier containing 2 platinum electrodes; a thermometer and mercury contact thermostat; insulated demineralizing chamber and polyethylene tubing connections to the constant temperature bath; actuating relay connecting the water-bath pump to the thermostat; and power source with variable voltage.

demineralizing solution was maintained by this method within ±1 C. of the desired temperature.

2. Samples of bone for the experiments were dense cortical bone from the shaft of an air-dried, adult, human femur. The chemical analysis of this standard bone was previously reported.[2]

3. One hundred milliliters of an acid solution of known molarity were used for demineralization.

4. Determinations of concentration of acid, calcium, phosphorus, and nitrogen were made on all solutions before and after demineralization. The technical methods used were described in the previous paper.[2]

5. Samples of bone were placed in the coil of the anodal electrode and moved through the solution at 25 r.p.m., and x-ray films of the samples of bone were made during the process of demineralization. When the samples were removed to be weighed or x-rayed, they were blotted on bibulous paper by the same investigator each time, and the weighings were made by the other investigator in order to keep errors constant. Repeated drying and weighings indicated that this error was not more than ±1 mg.

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Experiment 1: To determine if demineralizing conditions in the electrolysis machine are comparable to those previously used. Two pieces of the standard bone were shaped to approximately the same weight and surface area: Sample 1 weighed 232 mg., and had a surface area of 1.65 sq. cm.; Sample 2, 230 mg., and 1.63 sq. cm. The samples were demineralized separately in the electrolytic machine, *without electrolysis*, in 1 M hydrochloric acid for 2 hours at 25 C., after which the bone was weighed. X-ray films were also made, and the depth to which mineral had been removed was measured from the x-ray negatives. At the end of the experiment, the samples were digested in 25 ml. of concentrated hydrochloric acid, and the content of calcium, phosphorus, and nitrogen was determined.

Results. During the process of demineralization Sample 1 lost 53 mg. (23 per cent), and Sample 2 lost 49 mg. (21 per cent). The amount of mineral removed from the 2 samples differed by 1 mg., *i.e.*, 34 mg. of calcium and 12 mg. of phosphorus were removed from Sample 1, and 33 mg. of calcium and 12 mg. of phosphorus were removed from Sample 2. There was no difference in the amount of nitrogen removed, or in the depth to which mineral was removed from the 2 specimens.

The weight of mineral determined after digestion of the 2 pieces of bone differed by 1 mg., *i.e.*, 29 mg. of calcium and 11 mg. of phosphorus in Sample 1, and 30 mg. of calcium and 11 mg. of phosphorus in Sample 2. The total amount of mineral in each sample of bone was the same.

The reduction in the molarity of the acid during demineralization of the samples was so low that it was regarded as nil.

Experiment 2: The effect of electrolysis upon the rate of demineralization with hydrochloric acid. Two pieces of standard bone were shaped to approximately the same weight and surface area for demineralizing at 25 C., with and without electrolysis (Experiment 2A). The electrolytic sample weighed 185 mg., and had a surface area of 1.56 sq. cm., and the control sample, 174 mg., with the same surface area. Two more samples of standard bone were cut for demineralization at 45 C., with and without electrolysis (Experiment 2B). The electrolytic sample weighed 193 mg., and had a surface area of 1.67 sq. cm., and the control sample weighed 191 mg., and had a surface area of 1.66 sq. cm.

The bone was demineralized for 2 hours in 1 M hydrochloric acid at 25 C. or at 45 C. The electrolytic samples were treated at 5 volts and 3 amperes to provide a 15-watt electrolysis. Changes of acid, weighings, and x-ray films were made every half hour. The depth to which mineral had been removed was measured on the x-ray negatives.

Results. The data are summarized in Table 1. After 2 hours of demineralization at 25 C. (Experiment 2A), the sample treated by electrolysis lost 47 mg., and the control lost 48 mg. The amount of mineral removed from the electrolytic sample was 43 mg., and from the control sample 42 mg. Thus, the electrolytic and control samples differed by only 1 mg. with respect to loss in weight and the amount of mineral that was removed.

In Experiment 2B, after 2 hours of demineralization at 45 C., the sample

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TABLE 1

TOTAL LOSS OF WEIGHT, PER CENT OF WEIGHT LOST, AND TOTAL AMOUNT OF CALCIUM AND PHOSPHORUS REMOVED FROM BONES OF SIMILAR WEIGHT AND SURFACE AREA AFTER 2 HOURS IN 1 M HYDROCHLORIC ACID AT 25 C. AND 45 C., WITH AND WITHOUT ELECTROLYSIS

(Experiment 2A and 2B)

Time	Electrolysis Sample (15 watts)					Control Sample				
	Loss of weight	Per cent of weight lost	Mineral removed			Loss of weight	Per cent of weight lost	Mineral removed		
			Ca	P	Ca + P			Ca	P	Ca + P
Experiment 2A (25 C.)										
hr.	*mg.*		*mg.*	*mg.*	*mg.*	*mg.*		*mg.*	*mg.*	*mg.*
0	(185)					(174)				
2	47	25	30	13	43	48	27	31	11	42
Experiment 2B (45 C.)										
0	(193)					(191)				
2	69	36	42	14	56	66	35	39	14	53

treated by electrolysis had a loss in weight of 69 mg., and the control sample lost 66 mg. The amount of mineral removed from the electrolytic sample was 56 mg., and from the control sample 53 mg. Thus, the 2 samples differed by 3 mg. with respect to the loss in weight or the amount of mineral removed.

The molarity of the acid was determined at the end of each half hour. The reduction in the concentration of acid in the electrolysis solutions was from 1.0 to 0.7 M at 25 C., and from 1.0 to 0.6 M at 45 C.; the reduction in molarity of both control solutions was from 1.0 to 0.99 M. There was no difference in the amount of nitrogen removed from the electrolysis and control samples, and the depth to which mineral was removed was the same for both electrolysis and control samples.

Experiment 3: The effect of electrolysis on the rate of demineralization with phosphoric acid. Two pieces of standard bone were shaped to approximately the same weight and surface area for treatment with phosphoric acid at 25 C., with and without electrolysis (Experiment 3A). The sample for electrolysis weighed 134 mg., and had a surface area of 1.26 sq. cm., and the control sample, 130 mg. with a surface area of 1.28 sq. cm. Two more pieces of standard bone were cut for treatment at 45 C., with and without electrolysis. The electrolytic sample weighed 141 mg., and had a surface area of 1.32 sq. cm., and the control specimen weighed 142 mg., and had a surface area of 1.34 sq. cm.

The bone was demineralized for 3 hours in 1 M phosphoric acid at 25 C. or at 45 C. The electrolysis samples were treated with 10 volts at 1.5 amperes to provide a 15-watt electrolysis. Changes of acid, weighings, and x-ray films were made at intervals of 30 minutes. The depth to which mineral had been removed was measured from the x-ray negatives.

Results. During the 3 hours of demineralizing at 25 C. (Experiment 3A),

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the electrolysis sample lost 24 mg. (18 per cent of the original weight), and the control lost 22 mg. (17 per cent of the original weight). Thus, the difference in the amount of weight lost was 2 mg. During the 3 hours of demineralization at 45 C. (Experiment 3B), the electrolytic sample lost 31 mg. (22 per cent of the original weight), and the control lost 28 mg. (20 per cent of the original weight). Thus, the difference in the amount of weight lost by the 2 samples was 3 mg. Determinations of the amount of minerals lost are not reported inasmuch as the large amount of phosphorus in the solutions made the results of doubtful value.

The reduction in the molarity of the acid during demineralization was so small as to be within the error of measurement. There was no difference in the amount of nitrogen removed from the electrolysis and control samples, and the depth to which mineral was removed was the same for both electrolysis and control samples.

Experiment 4: The effect of electrolysis upon the rate of demineralization with formic acid. Two pieces of standard bone were shaped to approximately the same weight and surface area. The bone to be treated with electrolysis weighed 404 mg., and had a surface area of 2.33 sq. cm., and the control specimen weighed 401 mg., with a surface area of 2.37 sq. cm. Both pieces of bone were demineralized in 1 M formic acid at 25 C. for 3 hours. During the demineralization the one piece was also treated with 12 volts at 0.5 ampere to provide a 6-watt electrolysis. Weighings and x-ray films were made at the end of the 3 hours, and the depth to which mineral was removed was measured from the x-ray negatives.

Results. Both samples of bone decreased in weight by the same amount, *i.e.*, 7 mg. each, representing a loss of 2 per cent of the original weight. The same amounts of mineral, *i.e.*, 21 mg. (14 of calcium and 7 of phosphorus), were removed from both of the specimens, and the depth to which mineral was removed was the same.

The amount of mineral removed from each of the pieces of bone was greater than the loss in weight. This occurs when mineral acids of low molarity (below 0.5 M) or weakly ionizing acids are used for demineralization, and it is the result of hydration of the matrix of the bone.[2]

Reduction in the molarity of the acid solutions during demineralization was within the error of the titration for both the electrolysis and control samples, and there was no difference in the amount of nitrogen removed from the 2 specimens of bone.

DISCUSSION

The theoretical basis of the electrolytic method of demineralization is that a polarizing current will cause an increased rate of migration of the mineral ions from the sample of bone to the electrical pole of opposite charge. During demineralization, the primary mineral element to be removed from bone is the divalent, positively charged calcium ion. The minerals of bone can not be removed any faster than they are released from their combination within the

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bone, and the removal of the minerals, under any circumstances, can not be more rapid than the breakdown of the mineral complex by the demineralizing acid. In order to break down the mineral complex in bone, the acid must diffuse through the demineralized bone matrix and come in contact with the mineral complex. Hydrogen ions and calcium ions are positively charged. It is difficult to understand how a polarizing current can cause 2 positively charged ions to migrate in opposite directions, *i.e.*, the hydrogen ion into the bone, and the calcium ion out of the bone.

On theoretical grounds, it must be concluded that the electrolytic method of demineralization does not accomplish its purpose, inasmuch as (1) minerals can not be removed faster than the minerals are released from their complex within the bone, and (2) the electrolysis, rather than increasing the diffusion of the acid into the bone, probably tends to the opposite result. Thus, if the electrolytic method of demineralization does decrease the time of demineralization, the effect results from some cause other than the applied polarizing current.

For the present experiments, the range of difference in the loss of weight and the amount of mineral removed from paired samples of standard bone had to be determined under the conditions used in the electrolytic machine. In previous experiments under standard conditions,[2] the variation in loss of weight per unit of time was from 0 to 5 mg. for approximately matched bone samples. This difference (5 mg.) results from the difficulty in shaping pieces of bone to identical weights and surface areas; the slight differences in surface area, which often are beyond the range of accurate measurement, can lead to the small differences found in the experimental results.

Experiment 1 was made to determine if the difference in loss of weight and amount of mineral removed from matched samples of standard bones was the same in the electrolytic machine as that found under the standard conditions previously reported.[2] The data indicated that the difference in loss of weight was 4 mg., and the difference in the amount of mineral removed from matched samples was 1 mg. The difference in surface area of the 2 samples was 0.02 sq. cm.; the bone with the greater loss in weight had the greater surface area. These results were comparable to the differences found under the previously established standard conditions.

In Experiment 2, the loss in weight and the amount of mineral removed from matched samples of bone were determined with and without electrolysis in 1 M hydrochloric acid. At 25 C. the samples differed in loss of weight by 1 mg. when the surface areas were the same; at 45 C. the samples differed by 3 mg. where the surface areas differed by 0.01 sq. cm. The 3 mg. greater loss at 45 C. was in the bone with the greater surface area. Thus, the samples of bone demineralized with equal rapidity, with or without electrolysis, if all other conditions were made constant.

During electrolysis with hydrochloric acid, hydrogen chloride gas evolved, thereby decreasing the concentration of acid, as indicated by titrations of the remaining acid. Inasmuch as such a loss of acid might be a possible cause of the lack of effect of the electrolytic method with hydrochloric acid, 2 acids that

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do not form gas were used, *i.e.*, phosphoric and formic acids. In Experiments 3 and 4 the differences between the electrolytic and control samples were within the expected range of variation. There was no difference in the loss of weight from the 2 samples when formic acid was used, and, although there was a 3-mg. difference in loss of weight when phosphoric acid was used, the greater loss was noted in the sample that had a larger surface area. Thus, the loss of hydrochloric acid during electrolysis did not affect the time necessary for demineralization. Results from previous experiments[2] indicated that the reduction of concentration of hydrochloric acid from 1.0 to 0.6 M does not appreciably affect the rapidity of demineralization.

Electrolysis produces heat in the acid solution because of the resistance of the solution to the flow of current. Experiments with the electrolytic machine used in these studies revealed that a rise in temperature of 0.1 C. was produced per minute per watt of electrolysis in 100 ml. of 1 M hydrochloric acid. The amount of heat produced will vary with the resistance of the solution, but 1 M concentrations of other acids (*i.e.*, phosphoric, formic, and trichloracetic) had approximately the same rise of 0.1 C./minute watt 100 ml. of solution during electrolysis. In the previous paper,[2] the influence of temperature on demineralization was determined, and it was found that an increase of 20 C. (an increase from 25 to 45 C.) decreased by 15 to 20 per cent the time necessary for demineralization.

In the present experiments, an increase in temperature also decreased the time required for demineralization. Owing to the fact that the samples of bone used in the various experiments were of different weights and surface areas, and, in order to make proper comparison, the data should be expressed as milligrams of weight lost per square centimeter of surface area. The results of Experiment 2 indicate that bone demineralized for 2 hours in 1 M hydrochloric acid at 45 C. lost 0.40 mg. per sq. cm.; whereas the piece treated similarly, but at 25 C. lost 0.30 mg. per sq. cm. Thus, the difference was 0.1 mg. per sq. cm. of surface area. Similarly in Experiment 3, bone demineralized for 3 hours in 1 M phosphoric acid at 45 C. lost 0.23 mg. per sq. cm.; whereas the specimen treated at 25 C. lost 0.18 mg. per sq. cm., a difference of 0.05 mg. per sq. cm. Thus, the greater loss of weight that occurred at the higher temperature was in approximately the same range as that previously determined, and the time required for demineralization was reduced approximately 20 per cent.

The experiments described in this paper indicate that the electrolytic method of demineralization will result in higher temperatures in the acid solutions. At 15-watts electrolysis, the temperature of 100 ml. of 1 M hydrochloric acid will increase 15 C. in 10 minutes. The demineralization will then proceed at 40 C. or higher. If the time required for demineralization at 40 C. is compared with the time usually required at 25 C., the electrolytic method seems to be more rapid, but this is the result of the increased temperature of the demineralizing agent. When the temperature of the solution was controlled, the electrolytic and control samples demineralized at the same rate. Electrolysis produces heat in the acid solution, but electrolysis *per se* is not effective as a demineralizing agent

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SUMMARY

1. The use of electrolysis to bring about more rapid demineralization of bone is based on an unsound theoretical concept; namely, that 2 positively charged ions migrate in different directions in response to the same polarizing current, *i.e.*, the hydrogen ion into the bone, and the calcium ion out of the bone.

2. Electrolysis *per se* has no effect on the time required for demineralization.

3. Heat is produced in electrolytic baths, and an increase in the temperature of the demineralizing solution will result in less time being required for demineralization of bone. The controlled experiments described in this paper indicate that the reported decreases in time required for demineralization when using the electrolytic method are the result of higher temperatures caused by the electric current passing through the demineralizing solution.

SUMMARIO IN INTERLINGUA

1. Le uso del electrolyse con le objectivo de accelerar le dismineralisation de ossos es basate super un insolide concepto theoric, *i.e.*, le concepto que 2 iones a carga positive migra in differente directiones sub le effecto del mesme currente polarisante: le ion hydrogenic a in le osso, le ion de calcium a ex le osso.

2. Electrolyse per se ha nulle effecto super le tempore requirite pro le dismineralisation.

3. Calor es generate in le banio electrolytic, e un augmento del temperatura in le solution dismineralisante resulta in un reduction del tempore requirite pro le dismineralisation de ossos. Le experimentos controlate que es describite in le presente articulo indica que le reportate reductiones del tempore requirite pro le dismineralisation quando le methodo electrolytic es usate, resulta del elevate temperaturas que es causate per le electricitate currente a transverso le solution dismineralisante.

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STUDIES ON DEMINERALIZATION OF BONE

III. THE EFFECT OF ION EXCHANGE RESINS AND VERSENATE IN DEMINERALIZATION

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Recently attempts were made to decrease the time required for demineralization of bone by adding to the demineralizing acid various compounds that bind and remove the dissolved minerals of the bone. The compounds used for this purpose were ion exchange resins and chelating agents. Inasmuch as the action of these compounds is based on the same principle, they were considered as a unit in this investigation of their usefulness.

Dotti, Paparo, and Clarke[3] introduced the use of an ion exchange resin (Win 3000) mixed with formic acid. The method was said to result in demineralization more rapidly than other methods, and not to alter the morphology or staining reactions of the tissue. Lillie,[4] in a review of this paper, questioned whether the results of the method were actually better than formic acid alone because there was no control for comparison. Case[2] studied the use of a cation exchange resin in demineralization, and found that the addition of Win 3000 to formic acid did not appreciably shorten the time of demineralization, or enhance the preservation and staining qualities of the tissue.

Sreebny and Nikiforuk[7] and Birge and Imhoff[1] introduced a method of demineralization in which the chelating compound, ethylene diamine tetra acetate (or Versenate*), is used in acid solution. The method was admittedly slower than others, but it was thought to be useful because of the excellent preservation of the tissue.

The purpose of this study is to determine whether or not ion exchange resins or Versenate have advantages that are not found in the use of an acid solution alone.

MATERIALS AND METHODS

1. Samples of bone for the experiments were either dense cortical bone from the shaft of an air-dried, adult, human femur, or whole caudal extremities (bone and soft tissues) of male Wistar rats that were more than 150 days of age. The samples of human bone were from the standard sample of bone previously described (Morris and Benton[5]).

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* Versenate is the proprietary name assigned to this compound by the Bersworth Chemical Company, Framingham, Massachusetts.

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2. All demineralizations were made in polyethylene containers and the samples were agitated at 78 r.p.m. at 25 C. on a roller agitator.

3. One hundred ml. of acid solution of known molarity were used in each demineralization. In the experiments with resins 25 Gm. of resin (in the saturated hydrogen form) were added to 100 ml. of acid solution of known molarity. Four ion exchange resins were used for the experiments: IRC 50, a carboxylic acid type of cation exchanger that operates in a pH range from 4.0 to 8.0; IR 112, a sulfonic acid type of cation exchanger that operates over the entire range of pH; IR 120, a sulfonic acid type of cation exchanger that operates over the entire range of pH; Win 3000, an ammonium salt of a sulfonic acid type of cation exchanger that operates in the acid range.*

5. The extent of demineralization was determined by measuring the zone in x-ray films.

6. Determinations of concentrations of acid, calcium, phosphorus, and nitrogen were made on all of the solutions before and after demineralization. The technical methods were described in the section on Materials and Methods in a previous paper (Morris and Benton[5]).

EXPERIMENTS

Experiment 1: Demineralization Using Ion Exchange Resins and Acid, Compared with That from Acid Alone

Five pieces of standard bone were shaped to approximately the same weight and surface area, according to the method described in a previous paper (Morris and Benton[5]). The weights of the bone samples varied from 235 to 244 mg., and the surface areas from 165 to 169 sq. mm. One piece of bone was placed in each of the following solutions: 1 M hydrochloric acid; 1 M hydrochloric acid plus 25 Gm. of IRC 50; 1 M hydrochloric acid plus 25 Gm. of IR 112; 1 M hydrochloric acid plus 25 Gm. of IR 120; 1 M hydrochloric acid plus 25 Gm. of Win 3000. The samples of bone were agitated in these solutions until completely demineralized, as determined by x-ray films.

After the samples were completely demineralized they were digested in concentrated hydrochloric acid. Determinations of calcium, phosphorus, nitrogen, and molarity were made on all of the demineralizing solutions, and also on the digestion solutions.

Results. The various samples were not demineralized in the same amount of time; those in the acid alone, and in the IRC 50 and acid were demineralized in 17 hours. The samples in acid plus IR 112, IR 120, or Win 3000 were demineralized in 19 hours.

The loss in weight for each of the bones was approximately the same, but the least loss was found in the samples that were demineralized in the acid alone, or in acid plus IR 112 or Win 3000. Each of these samples lost approximately 105 mg. The samples that were demineralized in acid plus IR 120 or IRC 50 had a

* Win 3000 is distributed by the Winthrop Laboratories (formerly Winthrop-Stearns, Inc.), 1450 Broadway, New York 18, New York.

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TABLE 1

DEMINERALIZATION OF STANDARD BONE IN HYDROCHLORIC ACID AND IN ACID WITH ION EXCHANGE RESINS

(Experiment 1)

Time of Demineralization	Demineralizing Solution	Weight of Bone	Loss in Weight	Per Cent of Weight Lost	Mineral in Solution		Nitrogen in Solution	Nitrogen in Demineralized Matrix After Complete Digestion
					Ca	P		
hr.		mg.	mg.		mg.	mg.	mg.	mg.
17	1 M hydrochloric acid	240	104	43	66	23	Less than 0.1	9.1
	1 M hydrochloric acid plus 25 Gm. IRC 50	244	112	46	59	21	Less than 0.1	9.5
19	1 M hydrochloric acid plus 25 Gm. IR 112	235	103	44	27	21	Less than 0.1	9.1
	1 M hydrochloric acid plus 25 Gm. IR 120	241	111	46	12	22	Less than 0.1	9.2
	1 M hydrochloric acid plus 25 Gm. Win 3000	238	105	44	11	22	Less than 0.1	9.5

loss of approximately 111 mg., or 6 mg. more than the other samples. There was no significant change in the molarity of the acid in the solutions during demineralization; determinations of nitrogen on the demineralizing solutions revealed less than 0.1 mg. of nitrogen for all samples. The amount of unbound mineral and other results are recorded in Table 1.

The determinations of nitrogen on the digestion mixture of the demineralized pieces of bone revealed no appreciable difference in the amount of protein hydrolysis between the acid and the acid solutions with resins. All determinations were within 2 parts per 1000 of each other. No calcium or phosphorus was detected in the digestion solutions.

Experiment 2: Demineralization with Acid and Ion Exchange Resins and with Acid Alone, Using Bone and Associated Soft Tissues

The caudal extremities of male Wistar rats more than 150 days of age were fixed by perfusion with 10 per cent solution of formalin, or Bouin's solution, or 5 per cent solution of iodine in methanol.

Four acids and combinations of 4 resins with the 4 acids were used in this experiment. The acids were 1 M concentrations of hydrochloric, formic, trichloracetic, and phosphoric acids, and each was used as a control solution of acid alone, and with 25 Gm. of IRC 50, or IR 112, or IR 120, or Win 3000 resin. Thus, a total of 75 variations of fixative with acid solution, or fixative with acid-resin solution were used.

Before the extremities were placed in the demineralizing solutions, the muscles were trimmed to a thickness of approximately 1 centimeter on all sides of the bone. Complete demineralization was determined by x-ray films of all of the pieces of bone except those fixed in iodine solution. The presence of the iodine

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in the tissues made the specimens opaque and masked the removal of the calcium from the bone. After demineralization, the extremities were sectioned for microscopic examination, and these results are reported in the following paper.

Determinations of calcium, phosphorus, nitrogen, and molarity of the acid were made on the demineralizing solutions before and after demineralization.

Results. The order of rapidity with which the various samples were demineralized may have been due to differences in the size of the extremities, or the amount of muscle, or both, although the specimens were selected at random. In general, the control solutions (acid alone) were most frequently the fastest in demineralizing activity, and acids with IRC 50 or IR 112 were usually second in order. Acid solutions with Win 3000 were slow as often as they were fast, and those with IR 120 were most frequently the slowest in demineralizing activity.

Changes in the molarity of the acid solutions were slight, being less than 0.2 M in the controls and solutions with IRC 50, and less than 0.1 M in the solutions with other resins.

Determinations of nitrogen revealed that the largest amounts of nitrogen were removed from specimens that were demineralized in trichloracetic acid, with phosphoric acid, and formic acid (in decreasing order) removing lesser amounts. The least amounts of nitrogen were removed by hydrochloric acid. The amount of nitrogen removed by a certain acid solution was the same with or without the presence of the various resins. The average amount of nitrogen removed by the 4 acids was as follows, in mg. per 100 ml.: trichloracetic, 28.2; phosphoric, 12.3; formic, 9.5; hydrochloric, 8.5.

Experiment 3: Comparison of Alkaline and Acid Versenate with 1 M Hydrochloric Acid as Demineralizing Agents

Samples of standard bone were shaped to approximately the same weight and surface area, as previously described (Morris and Benton[5]): sample 1 weighed 85 mg., and had a surface area of 106 sq. mm.; sample 2, 88 mg., with a surface area of 105 sq. mm.; sample 3, 85 mg., with 105 sq. mm. of surface area.

Sample 1 was placed in 1 M hydrochloric acid, sample 2 in 0.1 M (saturated solution) disodium dihydrogen Versenate at pH 4.8, and sample 3 in 0.1 M (saturated solution) tetrasodium Versenate at pH 8.8. The tetra-hydrogen form of Versenate was not used inasmuch as it is almost insoluble at the higher levels of pH. The extent of demineralization was measured on x-ray films, and determinations of nitrogen were made on all of the solutions.

Results. Sample 1 was demineralized in 4 hours (control), but 12 days were required to achieve the same result with sample 2. Sample 3 was not completely demineralized when the experiment was terminated, after 25 days of treatment in 0.1 M tetrasodium Versenate at pH 8.8. The amount of nitrogen in all solutions was nil.

DISCUSSION

The purpose in using an ion exchange resin for demineralization is 2-fold,[3] *i.e.*, to decrease the time required, and to decrease the amount of destruction

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of soft tissue by the hydrolytic action of the acid. Ion exchange resins accept and bind cations; it was thought that the gradient of diffusion of salts out of the bone would be increased if the calcium cations were removed, thereby decreasing the time required for demineralization. The use of Win 3000 was said to prevent morphologic alteration and to maintain the typical staining reactions of the cellular elements. The mechanism of this protection was not described by the innovators of the method.

All cation exchange resins do not accept the same amount of calcium ions per Gm. of resin. Titrations of resins with calcium ions revealed that IR 120 and Win 3000 were the most efficient acceptors of calcium, and approximately 28 mg. of Ca^{++} were bound per Gm. of resin. IR 112 was intermediate in capacity, binding approximately 18 mg. of Ca^{++} per Gm., and IRC 50 was least, with less than 1 mg. of Ca^{++} per Gm. being bound in acid solution.

In Experiment 1 samples of standard bone were demineralized in mixtures of hydrochloric acid and resins. The results indicated that IRC 50 was the most rapid of the 4 resins tested as demineralizing agents, and this resin accepts the least amount of calcium ions among the group of 4 that were studied. In Experiment 2, where resins and acids were used for demineralizing bone (with associated soft tissues), IRC 50 and IR 112 were the most rapid of the resins as demineralizing agents, and these resins are relatively poor acceptors of calcium ions. On the other hand, resins with the greatest capacity for binding calcium ions were slower as demineralizing agents. In Experiments 1 and 2, the resin-acid mixtures did not demineralize bone as rapidly as did the acid alone.

Protection of the tissues by resins could not be demonstrated in these studies. The amount of nitrogen in the solution after demineralization is an indication of the amount of protein that is hydrolyzed by the acid solution. If the resin protects the soft tissues from hydrolysis by acid, the amount of nitrogen in the solutions of resins should be less than that found in the control acid solutions. In Experiment 1 the amount of nitrogen in the solutions after complete demineralization was the same for the acid control as for the acid-resin solutions, and the amount of nitrogen in the demineralized and digested samples of bone was the same, whether the specimens were treated with acid alone or with acid and resin. In Experiment 2, where the caudal extremities of Wistar rats were demineralized in different acids and various combinations of these acids with different resins, after different methods of fixation, the mass of muscle provided an abundant source of protein for hydrolysis by the acid. However, the amount of nitrogen removed from the pieces of bone was the same for all fixative-resin combinations with a given acid. There were differences in the amount of nitrogen released from the tissue, but these differences were related to the type of acid used, and not to the resin. Trichloracetic acid removed the most nitrogen from the tissues, and hydrochloric acid removed the least.

Versenate, a chelating agent, was relatively ineffective as a demineralizing agent, owing chiefly to its slow rate of activity. Acid Versenate did not have any advantages over hydrochloric acid, and it had the disadvantage of requiring 70 times as long to bring about demineralization. According to Sreebny and Nikiforuk,[7] one of the advantages of Versenate was that it could be used as a

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neutral or alkaline demineralizing agent. On the other hand, the results of Experiment 3 indicate, for all practical purposes, that demineralization did not occur in an alkaline solution of Versenate. Thus, in order to be effective, Versenate must be in the acid form, and then it has the disadvantages of any solution of a weak acid.

The authors previously demonstrated that the speed of demineralization is determined by the dissociation constant of the acid that is used to break down the mineral in the bone.[5, 6] If a resin is added to the demineralizing acid, it does not alter the amount of hydrogen ion that is present by accepting the calcium salts. Furthermore, the resin does not protect the tissue from the hydrolytic action of the acid by accepting the free cations. The resin may protect the tissue to a limited extent by removing the hydrogen ion from solution, and reducing the concentration of acid, but this was improbable in the experiments because the resins were in the saturated acid form initially. When they are in this state, resins will accept practically no additional hydrogen ion. This was demonstrated by titrations of the molarity of the acid before and after addition of the resins to the solution, and no difference in molarity was observed.

Hydrogen ion is the agent responsible for demineralization of bone, and for the alterations in morphology and staining qualities of the tissues. The resins and chelating agents are additives that remove calcium and other cations from the solution, but such substances are relatively ineffective as demineralizing agents.

SUMMARY

1. Four cation exchange resins (IRC 50, IR 112, IR 120, and Win 3000) were tested in acid solutions in order to determine their effect on demineralization of bone. The resins did not reduce the time required for demineralization below that required with the acid solution alone.

2. Ion exchange resins did not protect the soft tissues during demineralization. The amount of nitrogen removed from the samples was the same, with or without the addition of resins to the acid solutions.

3. Versenate was a weak acid type of demineralizing agent, similar in action to acetic acid. Although Versenate did not bring about destruction of tissues, it required a greatly increased amount of time for demineralization, and did not have any advantages over hydrochloric acid.

4. Versenate demineralized bone in alkaline solution extremely slowly.

SUMMARIO IN INTERLINGUA

1. Quatro resinas a excambio de cationes (IRC 50, IR 112, IR 120, e Win 3000) esseva probate in solutiones de acido pro determinar lor effecto super le dismineralisation de ossos. Le resinas non reduceva le tempore requirite pro le dismineralisation per le action del acido.

2. Resinas a excambio ionic non protegeva le histos molle durante le dismineralisation. Le quantitate de nitrogeno abferite ab le osso esseva le mesme sin e con le addition del resinas al solution de acido.

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3. Versenato esseva un dismineralisator del typo de acido extenuate. Su action esseva simile al action de acido acetic. Ben que Versenato non effectuava le destruction de histos, illo requireva grandemente augmentate intervallos ce tempore pro le dismineralisation e habeva nulle avantages in comparation con acido hydrochloric.

4. In solutiones alcalin, le dismineralisation de osso per le action de Versenato es extrememente lente.

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STUDIES ON DEMINERALIZATION OF BONE

IV. EVALUATION OF MORPHOLOGY AND STAINING CHARACTERISTICS OF TISSUES AFTER DEMINERALIZATION

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This paper deals with an evaluation of the morphology and staining characteristics of tissues after various methods of demineralization, as well as an analysis of (1) the effectiveness of certain fixatives in preventing destruction of tissue by hydrolysis with acid, (2) the staining reactions of cells and tissues, and (3) various methods of correcting poor staining reactions following the immersion of tissues in acid.

In order to make preparations of bone (with associated soft tissues) that are suitable for microscopic study, the bone must be demineralized before it can be cut into thin sections. The principle in demineralizing bone is to change the insoluble inorganic salts of bone to soluble salts that can be removed from the tissue. The use of acid solutions is the only presently known method that is effective in accomplishing this change.

The acid solutions frequently alter the morphology or staining reaction of the tissues associated with the bone, and, for this reason, several proposals have been made to modify acid demineralizing solutions in such a way that unwanted alterations will not occur. These methods may be grouped into 4 general categories:

1. The use of a weak acid for a long period of time (lactic acid, by Haug).[7]
2. The use of a strong acid for a short period of time (nitric acid, by Busch;[2] 50 per cent hydrochloric acid, by Ranvier).[12]
3. The use of protective agents mixed with various acids (phloroglucine with hydrochloric acid, by Ferreri;[6] alcohol with nitric acid, by Schaffer;[14] olive oil, alcohol, and picric acid, by De Galantha;[3] sodium citrate with formic acid, by Evans and Krajian).[5]
4. The use of exchange systems or absorbing agents to remove the bone salts from solution and increase the gradient of diffusion out of bone, thereby shortening the time required for demineralization (electrolytic method, by Richman, Gelfand, and Hill;[13] ion exchange resin, by Dotti, Paparo, and Clarke;[4] Versenate, by Sreebny and Nikiforuk,[15] and by Birge and Imhoff).[1]

Morris and Benton analyzed the relative efficiencies of various methods of demineralization under standard conditions.[9-11] The criteria for a good method

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of demineralizing bone were (1) rapidity of demineralization, and (2) a low amount of hydrolysis of protein, as demonstrated by only small quantities of nitrogen in the solutions after demineralization of the bone.

EXPERIMENTS

Experiment 1: Study of Smears of Blood and Bone Marrow under Various Conditions

The purpose of this experiment was to determine the effects of various acids, fixatives, and various concentrations of fixative and acids upon the preservation of cytologic detail and staining properties of blood cells in smears. These were prepared from human and rat peripheral blood and rat bone marrow by spreading a drop of blood or bone marrow in a thin film on a glass slide. The smears were stained for 1 minute in Wright's stain (Coleman and Bell, Cert. No. CWr-19) and diluted for 4 minutes with distilled water at pH 6.5, or they were stained for 2 hours in Giemsa's stain (National Aniline, BSC Cert. No. NGe-16) and differentiated in 0.1 M phosphate buffer at pH 7.0. Coverslips were not applied. These air-dried smears constituted the control samples for comparison with the experimental results.

The following experimental variables were employed:

1. Smears of peripheral blood and bone marrow were fixed in the following solutions for 2, 8, 12, and 24 hours and then stained with Wright's or Giemsa's stain: 10 per cent formalin; absolute methanol; absolute ethanol; Bouin's fluid; Bouin's fluid followed by 1 hour in 70 per cent ethanol; 5 per cent metallic iodine in absolute methanol; and 2 per cent aqueous osmic acid.

2. Fixed and unfixed smears of peripheral blood and bone marrow were immersed in 1 and 2 M concentrations of the following acids for 2 or 18 hours: hydrochloric acid; hydrobromic acid; phosphoric acid; trichloracetic acid; nitric acid; and formic acid.

3. After removal from the acid solution, the smears were (a) washed briefly in distilled water, or they were placed in Ringer's solution at pH 7.0 for 1 hour, prior to immersing in Wright's stain, or (b) they were placed in 0.1 M phosphate buffer at pH 7.2 for 1 hour, prior to immersing in Giemsa's stain.

4. All slides were marked with a code number and the results were analyzed without knowledge of the procedure that was used. Cytology and staining quality were judged separately, and the results were classified as *excellent*, *good*, *fair*, or *poor*. These classifications were defined before evaluation of the results as:

Excellent cytology. Preservation of the cytologic elements was equal to that in the control slides. The granules were clear, distinct, rounded, and evenly distributed. The cells were filled without being swollen, and were not shrunken or irregular. Nuclear and cellular membranes were distinct and intact. Nuclear chromatin was clear, and was situated either diffusely, or in blocks, or in blocks with threads, as was characteristic of the particular cell and fixative. Red blood cells contained hemoglobin, and were not shrunken or swollen.

Good cytology. Slight deviations from the excellent results were observed, such as indistinct or irregular granules, lack of clarity of the blocks or threads of

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nuclear chromatin, irregular distribution of elements within the cells, slight vacuolization of the cytoplasm, or slight shrinkage of the cells.

Fair cytology. The cells were shrunken or swollen, with indistinct clumps of granules or nuclear chromatin and large cytoplasmic vacuoles.

Poor cytology. The cells were characterized by destruction of granules, nuclear chromatin or cellular membrane, and there was gross shrinkage or swelling. The end point was complete disorganization of the cell.

Excellent staining. Coloring was equal to the best control slides. The granules were clearly stained with the proper dyes, and the cytoplasm was clear and lightly stained. Nuclear detail was clearly differentiated without staining of the nucleoplasm. The red blood cells stained red.

Good staining. There were slight variations in the balance of color from that regarded as excellent. The red blood cells were palely stained, or were slightly blue, or not stained at all. The background material of the nucleus and cytoplasm was overstained, or irregularly stained, and the granules had variations in the intensity of color.

Fair staining. Pale irregular staining was observed, with a tendency to overstain with either the red or blue component. Differentiation was incomplete owing to the overstaining of the cytoplasm and nucleoplasm.

Poor staining. Cells were stained with only 1 dye component, and were without differentiation. The background was heavily stained. In some instances cells were not stained, or they were stained with odd combinations of unusual colors.

Results. The results of the various combinations of fixative with acid with stain are summarized in Table 1.

1. Effect of fixation without acid immersion: Good morphologic preservation was obtained with all the fixatives tested. The optimal time for fixation was from 2 to 12 hours for blood smears; longer fixation made the cells more refractive to staining. Fixation in methanol, iodine in methanol, or ethanol (to a lesser extent) resulted in the best staining with Wright's or Giemsa's stain; smears fixed in 10 per cent formalin were always overstained with the blue component. Bouin's fluid without 70 per cent ethanol yielded poor results, with red blood cells that were yellow and loss of granules from the white cells; fixation in Bouin's fluid with 70 per cent ethanol eliminated the yellow color of the picric acid, and maintained the granules of the white cells, but the stain was still only fair. Staining following fixation with osmic acid was impossible owing to the binding of osmium to the cellular components, but morphologic detail following fixation with osmic acid was excellent.

2. Effect of immersion in acid: Unfixed smears were completely removed from the slides by immersion for 2 hours in any concentrations of the acids tested. With fixed smears no difference was observed between 1 and 2 M concentrations of hydrochloric, hydrobromic, and formic acids, and the cytologic results with these acids were good following fixation with 10 per cent formalin, Bouin's fluid with ethanol, iodine in methanol, and 2 per cent osmic acid. Distortion of cellular detail was noted in smears that were immersed in 2 M trichloracetic or phosphoric acids, but fair to good results were obtained in 1 M concentrations.

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TABLE 1

EFFECT OF FIXATION AND IMMERSION IN VARIOUS ACIDS ON THE CYTOLOGY AND STAINING OF BLOOD AND MARROW SMEARS*

(Experiment 1)

Fixative	Acid	Cytology	Wright's Stain	Giemsa's Stain	Fixative	Acid	Cytology	Wright's Stain	Giemsa's Stain
None	None	E	E	E	Bouin's fluid with 70 per cent ethanol	None	G	F	F
	HCl	Smears removed during immersion in acid				HCl	G	F	F
	HBr					HBr	G	P	P
	H_3PO_4					H_3PO_4	F	P	F
	CCl_3COOH					CCl_3COOH	F	F	F
	HNO_3					HNO_3	P	P	P
	HCOOH					HCOOH	G	F	F
10 per cent formalin	None	G	P	P	5 per cent iodine in methanol	None	G	G	G
	HCl	G	F	F		HCl	G	G	G
	HBr	G	F	F		HBr	F	F	F
	H_3PO_4	F	P	P		H_3PO_4	F	F	P
	CCl_3COOH	F	F	F		CCl_3COOH	F	F	G
	HNO_3	P	P	P		HNO_3	P	P	P
	HCOOH	G	F	F		HCOOH	G	G	G
Absolute methanol	None	E	E	E	2 per cent osmic acid	None	E	P	P
	HCl	F	G	G		HCl	E	P	P
	HBr	F	F	F		HBr	E	P	P
	H_3PO_4	F	F	F		H_3PO_4	E	P	P
	CCl_3COOH	F	G	G		CCl_3COOH	E	P	P
	HNO_3	P	P	P		HNO_3	F	P	P
	HCOOH	F	G	G		HCOOH	E	P	P
Bouin's fluid	None	F	P	P	Absolute ethanol	None	G	F	F
	HCl	P	P	P		HCl	F	P	P
	HBr	P	P	P		HBr	P	P	P
	H_3PO_4	P	P	P		H_3PO_4	P	P	P
	CCl_3COOH	P	P	P		CCl_3COOH	P	P	P
	HNO_3	P	P	P		HNO_3	P	P	P
	HCOOH	F	P	P		HCOOH	F	F	P

* E = excellent; G = good; F = fair; P = poor.

The results with nitric acid were always poor inasmuch as there was destruction of cytologic elements and loss of staining qualities.

In general, good preservation of cytologic features was maintained following immersion in acids if the smears were fixed in formalin, osmic acid, Bouin's fluid with 70 per cent ethanol, and iodine in methanol; fair preservation was obtained following immersion in acid after fixation in methanol, and there was poor preservation after fixation in Bouin's fluid alone or ethanol.

The staining characteristics of the cells were as good as the preservation provided by a given fixative. If the cells were well preserved, and the fixative did not interfere with good staining when there was no immersion in acid, the acid solution did not alter the results.

3. Description of cytologic results: Red blood cells had specific kinds of distor-

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tion of cytologic detail and staining quality, associated with particular combinations of fixatives and acids. The results were classified as follows: excellent—red blood cells similar to those in the standard smears; good—red blood cells weakly "basophilic" in staining reaction, but cytologically normal; fair—red blood cells pale in staining reaction and swollen; poor—red blood cells colorless and shadowy (membrane "ghosts"), or no cells present.

The basophilic staining reaction was a common result after immersion in acid, regardless of fixation. This was corrected by treating the smear for 1 hour in either Ringer's solution or 0.1 M phosphate buffer. After such treatment, the red blood cells were most resistant to immersion in acid following fixation in 10 per cent formalin or iodine in methanol. Fixation in Bouin's fluid with 70 per cent ethanol provided fair preservation, but Bouin's fluid alone yielded poor results, with colorless or shadowy cells. Fixation with ethanol or methanol resulted in little protection against immersion in acid, and the cells were pale and swollen.

Immersion of smears in hydrochloric, hydrobromic, and formic acids for 18 hours, after good fixation, did not bring about cytologic alterations of the red blood cells. The cells were usually pale and swollen after immersion in trichloracetic and phosphoric acids, regardless of the fixative, and the alteration was greater in 2 M than in 1 M concentration. Nitric acid destroyed the red blood cells.

The white blood cells manifested some resistance to alteration by immersion in acid. The most resistant were the lymphocytes, and then neutrophils, monocytes, and eosinophils in decreasing order. The ability of basophils to resist the effects of immersion in acid could not be determined owing to the limited number of cells. The blast cells in bone marrow were resistant to acid, but, as the cells became progressively more mature, they were more easily altered by the acid solution. The most labile elements were the cytoplasmic granules, and next there was swelling and alteration of the pattern of chromatin in the nucleus. Finally, there was fragmentation and complete disorganization of the cells.

Fixation for 12 hours in 10 per cent formalin, iodine in methanol, or Bouin's fluid with 70 per cent ethanol protected the white cells from cytologic damage during immersion in acid, but not to the same degree with all the acids. Best results were obtained with hydrochloric, formic, and hydrobromic acids. The cytoplasmic granules were indistinct after immersion of smears in 2 M trichloracetic acid, but 1 M concentrations did not cause any serious alteration. Phosphoric acid produced clumping and blue-staining of the cytoplasmic granules of the eosinophils and neutrophils. Nitric acid produced cells that were pale staining, swollen, or completely disorganized.

The platelets remained well preserved in all acids except nitric acid, and the best method of fixation was with iodine in methanol.

Experiment 2: Studies of the Preservation and Staining of Tissues Other than Peripheral Blood and Bone Marrow

The purpose of this experiment was to test the effect of fixation and immersion in acid on muscle and associated tissues.

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TABLE 2

EFFECT OF FIXATION AND IMMERSION IN ACID ON CYTOLOGY AND STAINING*
(Experiment 2)

Fixation	Acid	Cytology	Stain	
			Hematoxylin and eosin	1-step trichrome
Bouin's fluid with 70 per cent ethanol	None	E	E	E
	HCl	G	G	F
	HBr	P	P	P
	H_3PO_4	F	F	P
	CCl_3COOH	F	F	G
	HCOOH	G	G	G
10 per cent formalin	None	E	E	E
	HCl	E	E	G
	HBr	F	F	P
	H_3PO_4	F	G	P
	CCl_3COOH	G	G	G
	HCOOH	G	E	G
Absolute methanol	None	F	F	F
	HCl	P	P	P
	HBr	P	P	P
	H_3PO_4	P	P	P
	CCl_3COOH	P	P	P
	HCOOH	F	F	P
Iodine in absolute methanol	None	E	E	E
	HCl	E	E	G
	HBr	G	G	G
	H_3PO_4	F	F	P
	CCl_3COOH	G	G	G
	HCOOH	G	E	G

* E = excellent; G = good; F = fair; P = poor.

Muscle tissues from male Wistar rats more than 150 days of age were either perfused with, or submerged in fixing solutions. The animals were perfused by inserting a cannula into the dorsal aorta and washing the vascular system with Ringer's solution and then with the perfusion fluid. The skin was removed from the hind limbs of the rat, and the muscles were transected to make sections approximately 5 mm. thick. The entire limb (with the attached muscle) was then submerged in fixing solution for at least 24 hours. Following fixation, pieces of muscle, 5 mm. thick and approximately 1 cm. in the other dimensions, were cut from the limbs, washed in tap water, and placed in polyethylene containers with 100 ml. of the acid solution. The specimens were agitated at 78 r.p.m. on a roller agitator for 24 or 48 hours at 25 C. The pieces of muscle were then washed in water, dehydrated in graded solutions of ethanol, and embedded in paraffin with a melting point of 52 C. Sections were cut 7 microns in thickness and stained with hematoxylin and eosin or a 1-step trichrome stain (Morris).[3]

Five fixatives were used: 10 per cent formalin, Bouin's fluid, Bouin's fluid for 18 hours followed by 70 per cent ethanol for 6 hours, iodine in methanol.

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and methanol. Tissues fixed in these fluids were placed in either 1 or 2 M concentrations of the following acids: hydrochloric, hydrobromic, trichloracetic, phosphoric, formic, and nitric acids. Fixed tissues not immersed in the acid solutions served as the controls.

The slides were labeled in code and examined without knowledge of which procedure had been used. Evaluations were made according to preservation of morphologic detail, staining and balance of color, shrinkage, and friability of the tissue. All of the specimens treated in nitric acid were discarded, inasmuch as the connective tissue was digested, leaving the muscle as a macerated, structureless mass.

Results. The results are summarized in Table 2. Good preservation of the morphologic features and excellent staining were obtained in all of the control slides except those fixed in methanol. Bouin's fluid with 70 per cent ethanol, 10 per cent formalin, and iodine in methanol provided protection to muscle and connective tissue during immersion for 48 hours in hydrochloric, formic, and trichloracetic acids, but not during immersion in phosphoric and hydrobromic acids.

Iodine in methanol was an excellent fixative inasmuch as the fixed tissue seemed to be fresh. The muscle fibers were round, closely packed, and had brilliant and numerous nuclei. The shrinkage was minimal with this fixative. Fixing with formalin seemed to predispose the tissue to being "cooked" during embedding in paraffin. This was particularly true after immersion in hydrobromic, hydrochloric, and trichloracetic acids. If the tissue was allowed more than the minimal time for infiltration with paraffin, it would shred and fragment during sectioning. Bouin's fluid without 70 per cent ethanol resulted in poor staining of nuclei, and the connective tissue was swollen and partially digested following 48 hours of immersion in any of the acids tested. These disadvantages were eliminated by the additional fixation in 70 per cent ethanol.

Staining results paralleled the quality of fixation. If the fixation was good, the staining was good, but the tissue stained poorly in the center of the block where the fixation was imperfect. The staining of the nuclear chromatin with hematoxylin following immersion in the various acids was difficult, and this constituted the major alteration of morphologic detail in the series.

Experiment 3: Effects of Ion Exchange Resins and the Electrolytic Method on Preservation of Tissue

The caudal extremities of male Wistar rats more than 150 days of age were fixed by perfusion or immersion with 10 per cent formalin, Bouin's solution followed by 70 per cent ethanol, or 5 per cent iodine in methanol. Four acids and 4 cation exchange resins were used in the experiment. The acids were 1 M concentrations of hydrochloric, formic, trichloracetic, and phosphoric acids. Each acid was used in 5 ways: as a control solution of acid alone, and with 25 Gm. of

* Win 3000 is distributed by the Winthrop Laboratories (formerly Winthrop-Stearns Inc.), 1450 Broadway, New York 18, New York.

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TABLE 3

EFFECT OF FIXATION AND DEMINERALIZATION IN VARIOUS ACIDS AND ION EXCHANGE RESINS ON THE CYTOLOGY AND STAINING OF WHOLE LEGS OF RATS*

(Experiment 3)

Acid	Resin	Fixation in 10 Per Cent Formalin					Fixation in Bouin's Fluid				
		Cytology	H and E	Trichrome	Wright's	Giemsa's	Cytology	H and E	Trichrome	Wright's	Giemsa's
HCl	None	E	E	G	F	F	G	G	F	G	F
	IRC 50	E	E	F	P	F	G	G	G	P	F
	IR 112	E	E	G	F	F	G	G	P	F	F
	IR 120	E	E	F	F	F	G	G	F	F	F
	Win 3000	E	E	F	F	P	G	G	F	F	P
HCOOH	None	E	E	G	F	P	G	G	G	F	P
	IRC 50	E	E	F	F	P	G	F	F	P	P
	IR 112	G	G	F	F	F	G	G	G	F	F
	IR 120	G	G	F	F	P	G	G	F	F	F
	Win 3000	G	G	F	P	P	G	G	F	P	P
H_3PO_4	None	G	E	F	P	P	G	G	F	P	P
	IRC 50	G	G	F	P	P	G	G	F	P	P
	IR 112	G	G	F	P	P	G	G	F	P	P
	IR 120	G	G	F	P	P	G	G	F	P	P
	Win 3000	G	G	F	P	P	G	G	F	P	P
CCl_3COOH	None	G	G	F	F	F	G	G	F	P	P
	IRC 50	G	G	F	F	P	G	G	F	G	P
	IR 112	G	E	F	G	F	F	G	G	F	F
	IR 120	G	G	G	F	G	G	G	F	F	G
	Win 3000	G	G	F	P	F	F	G	F	P	F

* E = excellent; G = good; F = fair; P = poor.

IRC 50, or IR 112, IR 120, or Win 3000.* In all, 75 variations of fixative with acid with resin were used.

Before the extremities were placed in the demineralizing solutions, the muscle tissue was trimmed to approximately 1 cm. in thickness. Demineralization of the specimens of bone was done under standard conditions in polyethylene containers with 100 ml. of solution. The bone was agitated at 78 r.p.m. on a roller agitator at 25 C. until demineralization was complete, as determined by x-ray films.

Following demineralization, the tissues were washed in water, dehydrated in graded solutions of ethanol, and cleared in a 1:1 mixture of oil of wintergreen and xylene. The blocks were embedded in paraffin (melting point, 52 C.), and 10-micron sections were cut and mounted. The sections were treated in Ringer's solution at pH 7.2 for 2 hours, and were then stained with hematoxylin and eosin, 1-step trichrome, Wright's and Giemsa's stains.

Specimens were also demineralized in an electrolytic machine at 25 C., with 15 watts of electrolysis in 1 M hydrochloric acid, as described previously.[10]

Results. The histologic results from this study are summarized in Table 3, and

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the chemical data were reported in the preceding paper.[11] There was good preservation of structures and good staining when the tissues were fixed in 10 per cent formalin and demineralized in hydrochloric acid, with or without the addition of resins. Similarly, when tissues were fixed in 10 per cent formalin, treatment with formic acid also provided good slides, but those treated in trichloracetic acid were only fair, and consistently poor results were obtained after demineralization in phosphoric acid. Sections of tissue fixed in Bouin's fluid and 70 per cent ethanol were usually poorer than those fixed in formalin. There was a difference in clarity of differentiation and staining, rather than of morphologic identification of the cellular elements. Iodine in methanol resulted in poor preservation of the blood cells of the bone marrow, and these data are not recorded in Table 3.

The tissues that were demineralized in the electrolytic machine were similar to those demineralized in the acid without electrolysis; no differences between the control and experimental specimens could be demonstrated.

Experiment 4: Studies on Improved Methods of Staining Bone Marrow after Demineralization

The femora from male Wistar rats more than 150 days of age were removed, and the ends of the bones were split to expose the marrow cavity. The split bones were fixed in 10 per cent formalin or in Bouin's fluid and 70 per cent ethanol. The tissues were demineralized in 1 M concentrations of trichloracetic, hydrochloric, phosphoric, and formic acids, and in 0.1 M disodium dihydrogen Versenate. The specimens were placed in 100 ml. of the demineralizing fluid and agitated at 78 r.p.m. on a roller agitator at 25 C. until demineralization was complete, as determined by x-ray films.

After demineralization the specimens were washed in water, dehydrated in graded solutions of ethanol, cleared in 1:1 mixture of oil of wintergreen and xylene, and embedded in paraffin (melting point, 52 C.). Sections were cut 7 microns in thickness, mounted, and immersed in one of the following solutions for 1 hour prior to staining with Wright's or Giemsa's stain: phosphate buffer, pH 7.0; Ringer's solution, pH 7.0; Ringer's solution, pH 6.4; 2 per cent potassium dichromate in Ringer's solution, pH 9.0; 2 per cent potassium dichromate in Ringer's solution, pH 6.4; 5 per cent aqueous solution of magnesium chloride, or ammonium chloride, or sodium chloride; 2 per cent aqueous solution of sodium chloride; 5 per cent aqueous solution of barium chloride. The sections were cleared in butyl alcohol and xylene, and mounted in Clarite with coverslips.

Results. The results of the combinations of the variables in this experiment are listed in Table 4. Good cytologic detail was obtained after fixation in either 10 per cent formalin or Bouin's fluid with 70 per cent ethanol. Cellular staining comparable to that in the control blood smears was obtained with Wright's and with Giemsa's stain after demineralization in hydrochloric, formic, and trichloracetic acids, following the use of certain mordants.

Analysis of the data indicated that excellent results were obtained in 23 instances of the 200 different combinations of variables. These excellent results

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TABLE 4

EFFECT OF FIXATIVES, DEMINERALIZING AGENTS, AND MORDANTS ON THE CYTOLOGY AND STAINING OF BONE MARROW (Experiment 4)

Fixation	Acid	Cytology	Phosphate Buffer pH 7.0		Ringer's Solution pH 7.0		Ringer's Solution pH 6.4		2% $K_2Cr_2O_7$ and Ringer's Solution pH 9.0		2% $K_2Cr_2O_7$ and Ringer's Solution pH 6.4		5% Magnesium Chloride		5% Ammonium Chloride		5% Sodium Chloride		2% Sodium Chloride		5% Barium Chloride		Totals			
			W	Gs	W	Gs	W	Gs	W	Gs	W	Gs	W	Gs	W	Gs	W	Gs	W	Gs	W	Gs	E	G	F	P
10 per cent formalin	HCl	G	F	F	G	G	F	G	E	E	G	E	G	G	E	E	G	G	G	G	F	F	5	10	5	0
	HCOOH	G	P	G	F	G	G	F	G	G	G	G	G	G	E	E	G	G	F	F	F	F	2	11	6	1
	CCl_3COOH	G	F	G	G	G	F	G	G	G	G	G	G	G	E	E	G	G	G	F	F	F	2	13	5	0
	H_3PO_4	F	P	G	F	G	G	F	G	F	G	G	F	G	G	G	G	F	F	P	P	P	0	10	6	4
	Versenate	F	P	G	F	F	F	F	G	G	G	G	G	G	G	G	G	G	G	F	F	F	0	12	7	1
Bouin's fluid and ethanol	HCl	G	F	G	G	G	G	G	G	E	G	E	F	F	E	E	E	E	G	G	G	G	6	11	3	0
	HCOOH	G	F	F	G	G	G	G	G	G	G	G	F	F	E	E	E	E	G	G	G	G	4	12	4	0
	CCl_3COOH	F	F	G	G	G	F	G	G	G	G	G	F	F	E	E	G	G	F	F	F	F	2	10	8	0
	H_3PO_4	F	P	G	G	G	G	F	G	F	G	G	P	P	G	E	G	G	F	F	F	F	1	10	6	3
	Versenate	G	F	G	G	G	G	G	F	F	G	F	F	F	G	E	G	G	F	F	G	G	1	11	8	0
	Results:																									
	E								1	2		2			6	8	2	2					23			
	G			8	7	9	6	6	8	5	10	7	4	5	4	2	8	7	5	3	3	3		110		
	F		6	2	3	1	4	4	1	3		1	5	4				1	5	6	6	6			58	
	P		4										1	1						1	1	1				9

* Gs = Giemsa's stain; W = Wright's stain. E = excellent; G = good; F = fair; P = poor.

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occurred after immersion in a mordant of 2 per cent aqueous solution of potassium dichromate, or 5 per cent aqueous solutions of sodium chloride or of ammonium chloride. Poor results were obtained in 9 instances, *i.e.*, after immersion in phosphate buffer, in 2 per cent aqueous solution of sodium chloride, or in 5 per cent aqueous solutions of magnesium chloride or of barium chloride.

Phosphate ion was associated with every poor result, either as phosphate buffer or phosphoric acid. Tissues that were immersed in phosphate solutions were always overstained with the blue component of the dye, and had lack of staining with the red component. This sort of result was usually associated with Wright's stain, and occasionally with Giemsa's. Good staining was obtained with Giemsa's stain after treatment with phosphate buffer at pH 7.0, but excellent results were not obtained.

The analysis of the excellent results in the stains indicated that tissues treated with hydrochloric acid were excellent 11 times, whereas only 6 such instances were observed after treatment with formic acid, and 4 after use of trichloracetic acid. Preparations of tissue previously treated in phosphoric acid or in Versenate were excellent in only 1 instance each. Giemsa's stain provided excellent results 14 times, and Wright's in 9 instances, and excellent results in staining were observed 14 times after fixation in Bouin's fluid with 70 per cent ethanol, and 9 times after fixing in 10 per cent formalin. Similarly, excellent results were obtained in 14 instances after use of ammonium chloride, in 5 instances after use of potassium dichromate, and 4 times after 5 per cent aqueous solution of sodium chloride.

Thus, the best combination of factors for excellent staining of the cells in bone marrow seemed to be: (1) fixation in Bouin's fluid, followed by 70 per cent ethanol; (2) demineralization in 1 to 2 M hydrochloric acid; (3) use of 5 per cent aqueous solution of ammonium chloride as a mordant; (4) Giemsa's stain.

Experiment 5: Application of the Best Combination of Variables for Rapid Preparation of Sections of Bone Marrow

The following protocol represents a specific application of the results of studies to determine the most reliable rapid method for preparing excellent sections of decalcified bone:

1. Remove specimen of rib and place in fresh Bouin's fluid (approximately 2 hours).
2. Cut into cross sections with a Stryker bone saw (pieces approximately 1 cm. long, weighing approximately 2.5 Gm.). This type of section maximizes the area exposed to the demineralizing agent. Place specimens in fresh Bouin's fluid and treat on the roller agitator at 78 r.p.m. (approximately 4 hours).
3. Place specimen in 70 per cent ethanol and agitate again at 78 r.p.m. (approximately 2 hours).
4. Wash in tap water, and treat with 100 ml. of 1 M hydrochloric acid on the roller agitator at 78 r.p.m. at 25 C (approximately 3 hours).
5. Wash demineralized specimens in tap water and agitate at 78 r.p.m. during dehydration in graded solutions of ethanol (50, 70, 95, and 3 changes of absolute).

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Clear in 1:1 mixture of oil of wintergreen and absolute ethanol, then in 1:1 mixture of oil of wintergreen and xylene (approximately 12 hours).

6. Embed in paraffin (melting point, 52 C.) *in vacuo* and block; cut sections 4 microns in thickness, mount, and hydrate (approximately 2 hours).

7. Immerse sections in 5 per cent aqueous solution of ammonium chloride (approximately 30 minutes).

8. Apply Wright's stain, dehydrate, and clear in butyl alcohol and xylene; put coverslip in position with Clarite (approximately 15 minutes). If a Giemsa preparation is desired, apply the stain after step 7, differentiate, and coverslip after clearing in xylene (approximately $2^1/_2$ hours).

Results. There is practically no shrinkage of the tissue (Fig. 1), and the cytologic details are well preserved and vividly stained (Fig. 2). The total time required for preparation of slides stained with Wright's stain is approximately 26 hours, and for those stained with Giemsa's stain, approximately 28 hours.

DISCUSSION

The experiments on staining of bone and associated soft tissues (after demineralization of the bone) indicated that the critical element in the various procedures was the fixing of the tissues, *i.e.*, cytology was not altered by the acid, during demineralization, if the tissues were fixed well. A fixative that provides and maintains good preservation during demineralization should have the following characteristics:

1. The fixative should be relatively rapid in action owing to the fact that the cells in bone marrow undergo irreversible changes shortly after death or after removal of a piece of bone during surgery. The density of the bone and the limited amount of exposed surface make it desirable that the fixative penetrate well, in order that the deeper portions of the tissue may be preserved prior to disintegration of the cells.

2. The fixative should not harden the tissue excessively, inasmuch as further hardening that results from treatment in acid and embedding in paraffin may make it practically impossible to cut sections in which the histologic pattern is preserved.

3. The fixative should bring about precipitation of protein in an insoluble form. If the precipitate is not insoluble, treatment with acid during demineralization will result in excessive hydrolysis.

The advantages and disadvantages of various fixing solutions were observed in several experiments. Although Orth's, Zenker's, and Helly's fluids usually provide excellent preservation of cytologic structures, they were unsatisfactory for preserving bone marrow during demineralization. These fixatives were slowly penetrant and fixed only the outer millimeter or so of tissue; although this region had excellent preservation of cellular elements, the bone could not be used for study inasmuch as it was fragmented during the sawing of the specimen. In addition, dichromate fixatives made the tissue extremely hard, and after demineralization the sections tended to shatter and tear.

Metallic substances such as iodine (in methanol) and osmic acid provided

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excellent fixation of thin smears or tissues that actually came in contact with the fixing solution during perfusion. Following treatment with iodine in methanol, cells in the center of the block of bone were usually disorganized after demineralization because the iodine did not reach the deeper areas of the bone marrow with sufficient rapidity to bring about good fixation before autolysis began. Demineralization then resulted in digestion of the unfixed portions of the tissue. Osmic acid formed a complex with the proteins in such a manner that satisfactory staining of the tissue was impossible.

Solutions of alcohol were unsatisfactory fixatives because they did not protect the tissue from hydrolysis by acid. The nucleoproteins were well fixed, and the staining of nuclei was generally good after demineralization. Although the alcohols were rapidly penetrant, fixation was generally less effective in the center of the block than at the edge. The poorer fixation at the center of the block may have been the result of dilution by water absorbed from the tissue during the diffusion into the deeper portions. Alcohols did not harden the tissue excessively, but the precipitated protein was easily hydrolyzed by the acid solutions used for demineralization.

Formalin was the best general fixative. It was rapid in penetration, did not harden the tissue excessively, and the protein compounds formed were resistant to hydrolysis by acid. It was characteristic to have only small amounts of nitrogen in the demineralizing solutions after use of this fixative.

Bouin's fluid was rapidly penetrant and did not harden the tissue excessively. It was excellent for precipitating protein, but much of the precipitate was soluble in water and was hydrolyzed or lost during immersion of the tissue in acid. Bouin's fluid alone did not fix the granules of the white cells or the nuclear chromatin, and these elements did not resist hydrolysis during demineralization with acid. The use of ethanol as an additional fixative rendered these elements resistant, and fixation with Bouin's fluid followed by ethanol provided excellent preservation that was maintained during demineralization.

Various workers reported in the past that blood cells stained poorly after fixation with Bouin's fluid or formalin. For this reason other fixatives were used in order to obtain better results with staining, but such fixatives were not as suitable for tissues that were to be demineralized, inasmuch as the tissues were not resistant to damage by acids. In order to correct this deficiency, methods were proposed in which such substances as alcohol, olive oil, citrate salts, or phloroglucine were added for the purpose of protecting tissue from alterations by the acids. These additives depressed the ionization of the acids, and thereby led to a decreased amount of demineralization as well as to less alteration of the tissue. Similar results were obtained with a low concentration of acid, but some alteration of structures also occurred. In addition, low concentrations of acid resulted in excessive hydration of the tissue (Morris and Benton[9]), and this can predispose to poor results as do the higher concentrations of acid. These additives were not as effective in preventing alterations of tissue as was good fixation initially.

The addition of ion exchange resins to the demineralizing solutions did not

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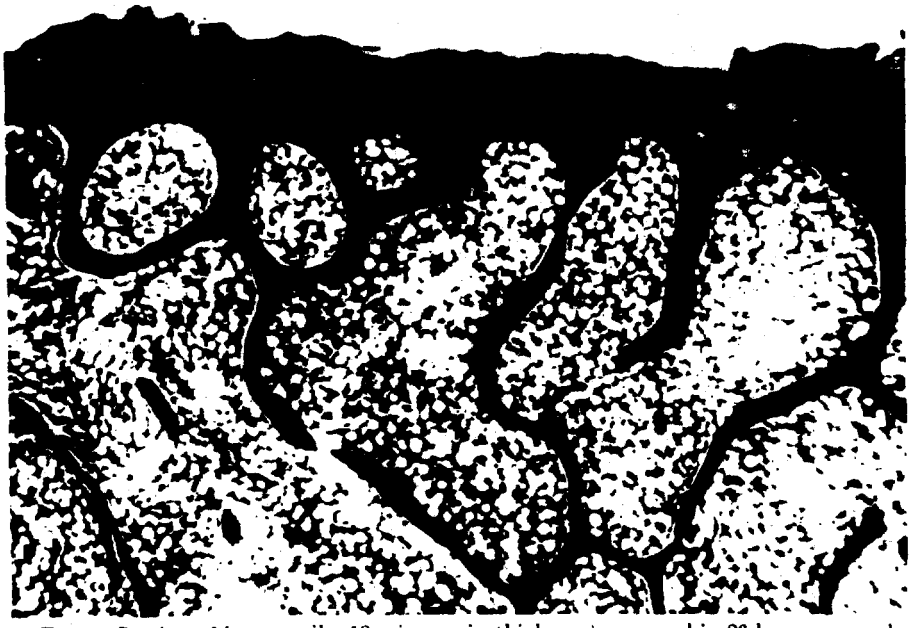

FIG. 1. Section of human rib (10 microns in thickness) prepared in 26 hours, according to the method outlined in Experiment 5. Note the *in situ* relations between elements of the bone marrow and the minimal shrinkage of the gross elements. Wright stain. Approximately × 20.

improve the preservation of the tissue or the quality of stained preparations obtained after demineralization. Furthermore, the electrolytic method of demineralization was no better than the same acid used at the same temperature without electrolysis. If the temperature of the acid was raised above 40 C. during electrolysis, the tissues seemed to be "cooked" and their staining reactions were poor. As a means of demineralizing bone, electrolysis had no advantages over those of hydrochloric acid alone.

After fixation in Bouin's fluid and ethanol or in formalin, staining reactions with Wright's and Giemsa's stains were improved by the use of mordanting solutions. The clarity of staining of cytoplasmic granules and the brilliance of red blood cells was restored by treating the slides in 2 per cent aqueous solution of potassium dichromate for 1 hour. Five per cent aqueous solution of sodium chloride also restored good staining, but the differentiation of granules was not as good as with dichromate solution. The best mordant was 5 per cent aqueous solution of ammonium chloride, inasmuch as the quality of staining was not distinguishable from that observed in air-dried smears of peripheral blood and bone marrow.

SUMMARY

1. The results of this study indicate that several methods of demineralization of bone are acceptable. Alterations that occurred in cytologic structures during

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demineralization in hydrochloric, formic, or trichloracetic acids were prevented by good fixation in 10 per cent formalin, or in Bouin's fluid followed by 70 per cent ethanol.

2. One to 2 M concentration of hydrochloric acid was the best demineralizing agent inasmuch as (a) it was the most rapid in action, (b) the greatest number of excellent staining reactions were observed after its use, and (c) it removed the least amount of nitrogen from the tissues. Equally good results were obtained less often after demineralization with formic acid, but it was considerably slower and greater amounts of nitrogen were removed from the tissues. Trichloracetic acid in 1 M concentration was an effective demineralizing agent, but a 2 M concentration brought about excessive alteration of cytologic structures.

3. Addition of ion exchange resins to the demineralizing solutions did not affect the process or improve the results obtained with the acid alone.

4. Treating sections with a mordant (2 per cent aqueous solution of potassium dichromate, or 5 per cent aqueous solution of ammonium chloride) restored staining quality to tissues that were fixed in 10 per cent formalin or in Bouin's fluid and 70 per cent ethanol. After use of either of these mordants, the quality of Wright's and Giemsa's stains on sections of tissue that were demineralized could not be distinguished from that observed in air-dried control smears of peripheral blood and bone marrow.

5. If one uses the best possible combination of factors that were studied in these experiments, bone may be fixed, demineralized, cut into histologic sections, mordanted, and stained with Wright's stain in 26 hours, or with Giemsa's stain in 28 hours.

SUMMARIO IN INTERLINGUA

1. Le resultatos del presente studio indica que plure methodos de dismineralisation de ossos es acceptabile. Alterationes que occurreva in structuras cytologic durante le dismineralisation in acidos formic, hydrochloric, e trichloracetic esseva prevenite per bon fixation in formalina a 10 pro cento o in fluido de Bouim sequite per ethanol a 70 pro cento.

2. Un concentration a 1 a 2 M de acido hydrochloric esseva le melior agente dismineralisante in tanto que illo esseva (a) le plus rapide in su action, que illo resultava (b) in le plus grande numero de excellente reactiones de coloration, e que illo abfereva (c) le plus parve quantitate de nitrogeno ab le histos. Resultatos equalmente bon esseva obtenite minus frequentemente post dismineralisation per medio de acido formic, sed iste agente esseva plus lente, e illo abfereva plus

FIG. 2. Section of human rib (4 microns in thickness) prepared in 28 hours, according to the method outlined in Experiment 5. The 2 photomicrographs illustrate the maintenance of basophilic cytoplasm and nuclear detail in the myeloblasts, and the granular detail in the promyelocytes. In the erythrocytic series there is proper balance of basophilia in the cytoplasm and early acidophilia of the hemoglobin in prorubricytes. Giemsa stain. Approximately × 1250.

Editor's Note: Cytologic details in the original preparations were even more distinct and vivid than the illustrations indicate. The printed reproductions were copied from colored negatives, and some detail was lost owing to the technical limitations.

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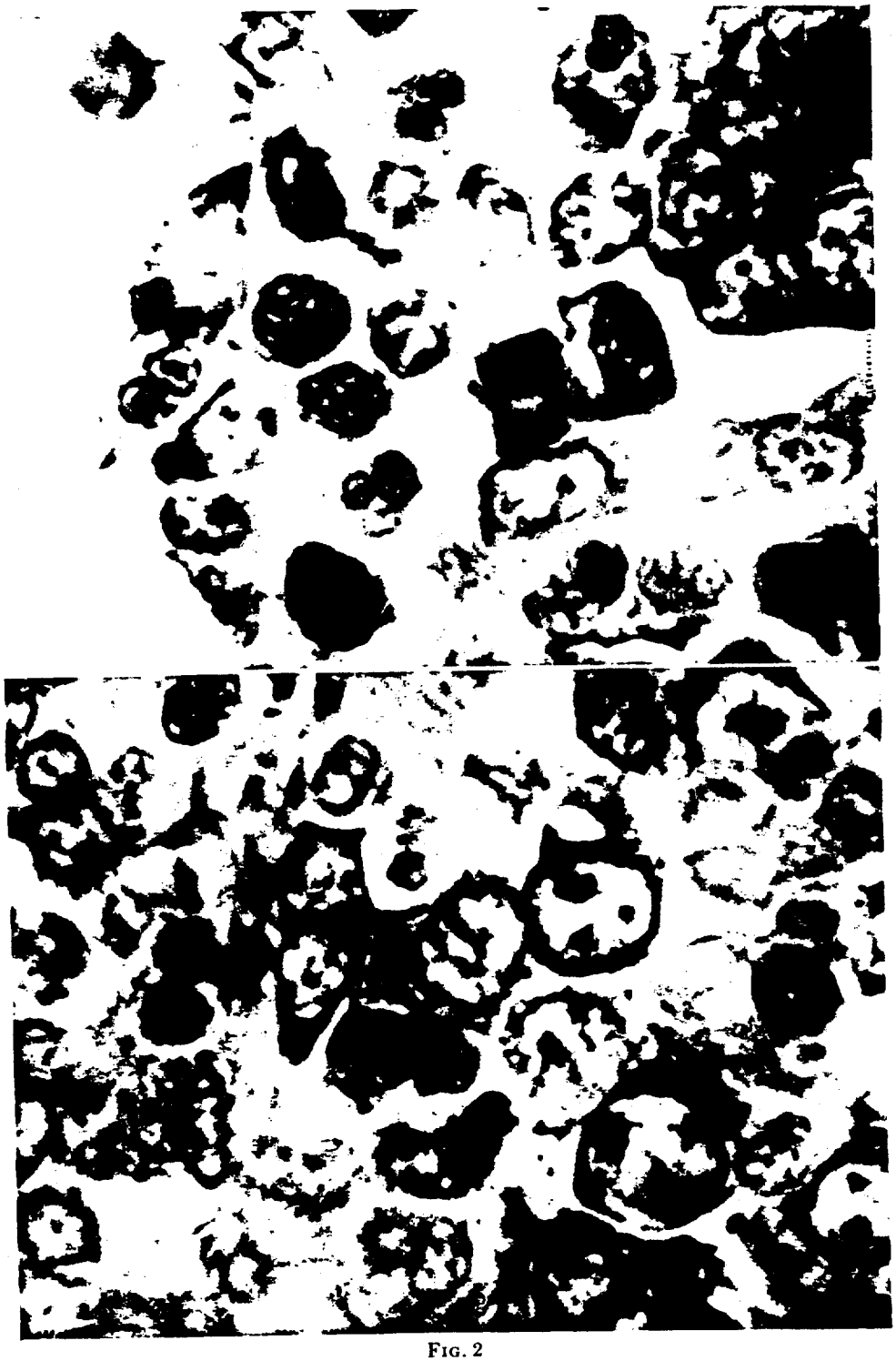

FIG. 2

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grande quantitates de nitrogeno ab le histos. Acido trichloracetic in un concentration de 1 M esseva un efficace agente dismineralisante, sed in un concentration de 2 M illo effectuava un excessive alteration del structuras cytologic.

3. Le addition de resinas a excambio ionic al solutiones de dismineralisation non afficeva le processo e produceva melior resultatos que le acidos sol. Le tractamento del sectiones con un mordente (2 pro cento de dichromato de kalium in un solution aquose o 5 pro cento de chlorido de ammonium in un solution aquose) restaurava le qualitates de coloration in histos fixate per formalina a 10 pro cento o in fluido de Bouin sequite per ethanol a 70 pro cento. Post le uso del un o del altere del mentionate mordentes, le qualitate del colorantes de Wright e de Giemsa in sectiones de histos dismineralisate non esseva distinguibile ab lo que esseva observate in aero-desiccate frottis de controlo de peripheric sanguine e medulla ossee.

5. Si on usa le melior possibile combination de factores studiate in iste experimentos, un specimen de osso pote esser fixate, dismineralisate, secate in sectiones histologic, tractate a mordente, e colorate con le colorante de Wright in 26 horas o de Giemsa in 28 horas.

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