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STUDIES OF BONE DEMINERALIZATION

Contract No. AT-(40-1)-1542

FINAL PROGRESS REPORT

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This report is intended to cover:

A) The first phase of a program of research to define efficient methods of demineralization of bone while preserving associated soft tissues. The methodology and some data have been reported previously in a Progress Report (under Contract No. AT-(40-1)-1542), submitted to the United States Atomic Energy Commission on September 15, 1953. Attention should be directed to this Report for explanation of the purposes and outline of the work of which this Report gives the conclusions.

B) Certain experiments and results which conclude the determination of the physical factors which appear to operate in any method of demineralization of bone. These experiments are:

1) An experiment to determine the effect of weight upon rate of demineralization, surface area being constant.
2) An experiment to determine the efficiency of certain acids in removal of mineral from standard bone samples and a determination of the amount of acid utilized in removing mineral from the standard samples.

With the reporting of these experiments, the first phase of this program is closed, we believe, because the work to date has accomplished its major purposes: a) to identify the physical factors basic to any demineralization process, and b) to test these factors under controlled conditions to determine something of the extent to which they influence demineralization.

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Investigation of the nature of the processes by which demineralization occurs, therefore, is beyond the scope of this study.

EXPERIMENTS AND RESULTS:

1. Experiment to determine the effect of weight upon rate of demineralization, surface area being constant.

This experiment was earlier reported in the Progress Report of September 15, 1953 (see pp. 4 and 5 of that Report). In the previous experiment it was found that the amount of mineral removed during some periods of the demineralization process exceeded the total weight loss of the bone sample. In subsequent experiments on the effect of acid concentration, it was found that acid of low molarity would produce this effect. It was suggested that this difference in weight might be due to the demineralized matrix taking up water in the presence of an acid solution of low ionic strength.

In order to produce conditions which would more nearly fit those of its companion experiment, that in which weight was held constant while surface area was varied (see: Progress Report, p. 3), this experiment was repeated using 0.5 molar hydrochloric acid rather than an acid solution of lower molarity.

Materials and Methods:

Samples were taken from the same part of the standard bone. They were cut on a band saw and shaped for surface area and weight on coarse and medium mill bastards. The forms used were the cylinder and the bar. In the two samples the surface

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areas were made equal, but the cylinder had a weight 44% greater than that of the bar.

Data:

	Bar	Cylinder
Weight	0.639 grams	1.129 grams
Surface area	4.60 sq. cm.	4.60 sq. cm.

The samples were placed in separate polyethylene containers, each containing 100 ml. of 0.5 molar hydrochloric acid. The containers were placed upon a roller agitator revolving at 85 rpm. Temperature during the experiment was controlled at 25°C. ± 1°. Weighings and changes of the demineralizing solution were made at pre-determined intervals. Calcium and phosphorus determinations were made upon each of the solutions. For the Data, see Tables I and II.

Results:

As can be seen from the data in Table I, the weight loss of the two bones was about equal throughout the experiment. The average difference between the two samples in weight loss was 4.3% -- within the error of the method of shaping the bones to surface area. Also, it is to be noted that the difference between the two bones in total mineral removed (Table II) during the 8-hour period is of the same order - 5%.

Upon comparison of these results with those of the experiment in which surface area was varied but weight was held constant, it can be seen that the difference is striking:

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	Surface area varied Weight constant	Weight varied Surface area constant
Difference in wt. loss between bar and cylinder	47%	4.3%

With reference to this experiment, the following points may be noted:

1. Varying the mass of the samples while holding the surface area constant did not change the rate of demineralization during the 8-hour period. This is further confirmation that surface area is a major limiting factor in demineralization.
2. The total weight of the mineral removed from the samples is approximately equal regardless of the mass of the samples.

2. Experiment to determine the efficiency in demineralization of certain acids.

The purpose of this experiment is to rank the acids more commonly used as demineralizing agents in the order of their efficiency as judged by rate of removal of mineral from standard bone samples. Earlier work of this kind was done on rat femora (Progress Report, pp. 21-23) which precluded the element of control obtainable by the use of samples from the standard bone. In addition, the weight loss curves for the bones in the various acid solutions did not exactly coincide with the amount of mineral determined to have been removed.

Materials and Methods:

The acids used were: Hydrochloric, Nitric, Hydrobromic, Phosphoric, Trichloroacetic, Formic, Lactic, Citric, and Acetic.

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The bone samples were taken from the same part of the standard bone. They were cut on a band saw and shaped for surface area and weight on coarse and medium mill bastards. The samples thus formed varied in weight between 0.400 grams and 0.434 grams and in surface area between 0.218 sq. cm. and 0.235 sq. cm.

The samples were placed in 100 ml. of a 1 molar concentration of the acids in polyethylene containers and placed upon a roller agitator revolving at 85 rpm. Temperature during the experiment was controlled at 25°C. ± 1°. Weighings and changes of the solutions were made at predetermined intervals. Calcium and phosphorus determinations were made upon each of the solutions. See Tables III and IV for these data. The amount of acid used in the demineralizing process was determined by titration against standard NaOH solution. (Table V).

At the end of the experiment, the samples were cleared in beechwood creosote and cedarwood oil. This rendered the demineralized portion of the bone translucent and permitted measurement of the depth to which the mineral had been removed.

Results:

The acids used have been ranked in the following ways below for comparison. The acid at the top of each column representing "most" and that at the bottom "least".

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Wt. Loss	Penetration	Mineral Removed	Solubility of Ca Salt	Dissociation
HCl	HCl	HCl	HNO_3	HBr
HNO_3	HBr	HNO_3	HCl	HNO_3
HBr	HNO_3	HBr	HBr	HCl
Trichloro.	H_3PO_4	Trichloro.	Trichloro.	Trichloro.
H_3PO_4	Trichloro.	H_3PO_4	Acetic	H_3PO_4
Formic	Formic	Formic	H_3PO_4	Formic
Lactic	Lactic	Lactic	Formic	Lactic
Citric	Citric	Acetic	Lactic	Citric
Acetic	Acetic	Citric	Citric	Acetic

In the above ranking, three divisions are seen in the acid groups: the strong mineral acids which are the most rapid demineralizers, the second group of acids which are primarily organic acids and moderately rapid demineralizers, and finally the weak organic acids which are slow demineralizers.

Several common features can be seen in each group. In the first group, the dissociation of the acid is of the order of $7\text{-}9 \times 10^{-1}$, and the solubility of the calcium salt at the experimental temperature is from 125 to 315 grams per 100 ml. of water. Thus, these acids have high solubility of the calcium salts formed, and a high degree of ionization which is necessary for the breakdown of the mineral in bone. These appear to be the characteristics of a rapid demineralizer.

In the second group, the ionization is reduced, being in

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the range of 2×10^{-1} to 1.76×10^{-4} and the solubility is of the range of 15 to 70 grams per 100 ml. of water. Thus, these acids are fair demineralizers in rate and are reduced in both ionization and solubility of the calcium salt.

Finally, the last group of acids have both low dissociation constants and low solubility of their calcium salts. The dissociation constants are in the range of 1.76×10^{-5} to 1.38×10^{-4}. The solubility of the calcium salts varies from 0.25 grams to 35 grams per 100 ml. of water.

From this experiment it may be posited that there are two factors involved in the demineralization process: (1) the rate of breakdown of the insoluble mineral within the bone into a soluble calcium salt which is a function of the dissociation constant of the acid; and (2) the removal by diffusion of the salt which is a function of the solubility of the calcium salts of the acid. If the relative rank of an acid in mineral removed be considered as the resultant of these two factors of dissociation constant (rate of breakdown) and solubility of calcium salt (rate of removal), it becomes more meaningful. For example, acetic acid has a solubility product of about 35 grams per 100 ml. of water of its calcium salt which is higher than that of calcium formate or calcium phosphate. Yet, in terms of mineral removed, it ranks much lower than either in the series. However, if the dissociation constant is considered, it is seen to be the lowest in the series of acids

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tested. Thus, the actual rank of acetic acid represents the result of some balance between these two factors and is not wholly determined by either.

Results of Titrations of Acids after Demineralization:

The acids used in the above experiment were titrated against standard NaOH solution to determine:

1. Whether an excess of acid had always been present about the bone samples.

2. How much acid was used in the demineralization process.

3. What the efficiency of the acid is in terms of the moles of calcium and phosphorus per hydrogen equivalent.

It was also expected that this might permit some estimation of acid action on components of bone other than mineral.

From the titration data (Table V), it is seen that only a small amount of acid was used in the actual demineralization. As has been previously reported (Progress Report, September 15, 1953), the optimal concentration of acid is from 0.5 to 2.0 molar. The titration data shows that the acid concentration always remained in this optimal range. The amount of acid used varied from 0.01 molar to 0.3 molar during the experiment. This amounts to a maximum use of 30% of the available acid in the case of citric acid (which removed only a trace of mineral), and an average of about 10% of the available acid for the rest of the acids.

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More informative are the following data which are calculated on the basis of the equivalents of hydrogen used to remove milligram-moles of calcium and phosphorus. The ratio of hydrogen equivalents to milligram-moles of mineral is expressed in the final column and the acids, in this case, are ranked on this basis.

Acid	Equivalents of Hydrogen used	Mg.-Moles of Calcium & Phosphorus Removed	Ratio of H to mineral
HCl	3.17	3.233	0.98 : 1
Trichloroacetic	1.93	1.709	1.12 : 1
Formic	1.07	0.855	1.20 : 1
H_3PO_4	2.01	1.105	1.81 : 1
HNO_3	9.73	2.869	3.39 : 1
HBr	19.48	2.697	7.22 : 1
Acetic	3.85	0.321	11.99 : 1
Lactic	11.29	0.528	21.38 : 1
Citric	33.58	Trace	?

On the basis of the above comparison, it is possible to establish a criterion of efficiency of an acid in removing mineral from bone. Thus, in hydrochloric acid, the units of acid used are about equal to the units of mineral removed. In this sense it is an efficient acid. Trichloroacetic, phosphoric, and formic also are relatively efficient in this sense, whereas nitric, hydrobromic, acetic, lactic, and citric show progressively larger amounts of acid expended per unit of mineral removed.

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This evaluation, it is to be noted, says nothing concerning rate, or other factors possibly involved, that would influence the usefulness of an acid in demineralization. Thus, while an acid such as Hydrobromic appears to be inefficient on the basis of the hydrogen equivalent - mineral removed ratio, on the basis of rate of removal of mineral it ranks well ahead of other acids which have a ratio much nearer unity. On the other hand, if an acid like nitric is removing almost as much mineral per unit time as is hydrochloric but is also using more than three times as many hydrogen equivalents to do it, the suspicion is aroused that something beside mineral is being actively attacked. This suspicion is reinforced when the amount of acid used is great and the yield in mineral small as in the case of lactic, acetic, and citric. In addition, the behavior of trichloroacetic acid introduces another factor. This acid in 2 molar concentrations has been observed to distort and warp rat femora left in the solution for a 24-hour period. Thus, while it appears to be relatively efficient according to the ratio presented above and intermediate in rate of removal of mineral, it has been shown to have other undesirable effects.

Ideally, an acid used in demineralization should have a low hydrogen equivalent to mineral removed ratio, a rapid rate of removal of mineral, and an absence of deleterious side effects. Thus far, it appears that hydrochloric acid most closely approaches this ideal.

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TABLE I

Time	Wt. Loss (Mgs.) Bar	Wt. Loss (Mgs.) Cylinder	Difference in Wt. Loss (as %)
1	66	71	8
2	99	101	2
3	122	127	4
4	143	146	2
5	159	167	5
6	177	183	4
7	188	198	5
8	201	211	5
			Av. 4.3

TABLE II

Solution	Calcium (Mgs.) Bar	Calcium (Mgs.) Cylinder	Phosphorus (Mgs.) Bar	Phosphorus (Mgs.) Cylinder	Total (Mgs.) Bar	Total (Mgs.) Cylinder
I	48	50	18	20	66	70
II	30	30	10	10	40	40
III	30	34	11	12	41	46
IV	29	28	10	11	39	39
	137	142	49	53	186	195

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TABLE III

Time (Hours)	% Weight Loss								
	Hydro-chlor.	Nitric	Hydro-brom.	Tri-chloro.	Phos-phoric	Formic	Lactic	Citric	Aceti
1	11	11	10	5	4	1	0.6	0.4	0.4
2	16	16	15	7	6	2	1	0.7	0.2
3	20	20	18	9	9	3	1	1	0.2
4	23	23	21	10	10	4	2	1	0.4
5	25	26	23	12	11	5	2	1	0.9
6	28	27	25	13	13	6	3	2	0.9
7	29	29	27	15	14	7	4	2	0.9
8	31	30	28	16	15	7	4	3	1

TABLE IV

Mineral Removed

	Calcium mg/100 ml.	Phosphorus mg/100 ml.	Total
Hydrochloric	88	32	120
Nitric	75	30	105
Hydrobromic	69	30	99
Trichloroacetic	42	20	62
Phosphoric	40	3	43
Formic	22	9	31
Lactic	16	3	19
Acetic	8	3	11
Citric	Trace*	0	

*The amount found in each solution was so small as to fall within the error of measurement of the method.

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Table V.

Titrations of the Acid Solutions Used in Demineralization

Acid	Molarity of Acid Solution	Molarity of Acid Solution after Demineralization	Grams of Acid Used in Demineralization
HCl	0.9123	0.8806	0.1156
HNO	1.0226	0.9253	0.6131
HBr	0.9541	0.7593	1.5763
Trichloro-acetic	0.9049	0.8856	0.3153
Phospheric	0.7261	0.6858	0.3951
Formic	0.9391	0.9284	0.0492
Lactic	0.9128	0.7999	1.0170
Acetic	0.9006	0.8621	0.2311
Citric	1.1744	0.8386	6.4513

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DISCUSSION OF THE RESULTS OF THE FIRST PHASE OF THE STUDY OF DEMINERALIZATION

Definition has been made of the limitations and the factors which influence the limitations in the demineralization of bone. These are:

Limitations:

1. The effect of surface area on the rate of demineralization.
2. The effect of diffusion distance upon the rate of demineralization.

Factors which influence the limitations:

1. The effect of agitation and lack of agitation upon the rate at which mineral is removed from bone.
2. The effect of variation in temperature upon the rate of removal of mineral from bone.
3. The effect of concentration of acid on the rate of removal of mineral from bone.
4. The relative efficiency of various acids in removing mineral from bone. This involves; a) the dissociation constant of the acid. b) the solubility of the salt of the acid.

Although these have been studied separately, the actual process of demineralization involves some relationship of each one to all of the others. The complexity of this relation leads to a recognition that there are practical limits to the control which can be exercised over the demineralization process. Thus, these experiments have attempted a) to identify the basic limitations that will apply to any process of demineralization, b) to explore the physical manipulations that might influence the basic limitations, and c) to define, within rough limits,

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the extent to which these limitations may be influenced.

The Limitations:

1. Surface Area: This forms a prime limitation upon the rate of demineralization since the amount of area available to attack by the demineralizing agent will define the maximum rate of removal of mineral under any set of conditions. In the regularly shaped bone samples of uniform composition such as were used in these experiments, the surface area decreased at a relatively uniform rate during greater part of the process. In situations where bones or parts of bones of more complex structure are used, the rate of decrease might not be uniform, as, for example, when the demineralizing agent, having removed the mineral from the cortex of a bone, finally reached the trabecular structure of the interior. However, no matter how complex the given situation, the same rule will apply to each of its parts - a constantly decreasing surface area defining a constantly decreasing rate of mineral removal.

2. Diffusion and Diffusion Distance: This is a problem which becomes increasingly important during the process of demineralization. The distance that materials must move becomes greater as mineral is removed from the more superficial parts of the bone, and this places an additional limitation upon that already determined by the decreasing available surface area.

Basically, there are three factors involved in diffusion and, in addition, many related variables which influence the

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basic factors. There is, first, a distance of diffusion inward through which the demineralizing agent must travel from the surface of the bone to, second, the site of breakdown where the mineral is being put into soluble form by the demineralizing agent. Finally, there is a diffusion distance outward of the dissolved mineral from the site of breakdown to the outer surface of the bone. As demineralization continues, both the inward and outward diffusion distances increase. The arrival of demineralizing agent at the point of breakdown is slowed, and the transport of the soluble salts away is, likewise, decreased. In addition, once the surface mineral has been removed, the amount of liquid available for ionization of the acid and transport of the soluble salts is limited by the demineralized matrix.

Factors which influence the limitations:

Since it is recognized that the surface area available to the demineralizing agent cannot be varied once demineralization is in progress without altering the specimen itself, and since the distance of diffusion for the various products cannot be altered, methods of increasing the rate of demineralization must influence the gradient of diffusion to bring about the most rapid exchange practicable. This involves the selection of an efficient acid and the manipulation of several physical factors.

1. The effect of agitation as a rate increaser in demineralization:

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Agitation of the demineralizing solution and the bone sample will increase the rate of demineralization by about 25%. This increase in rate is believed to be due to three factors:

a) It insures a constant renewal of the solution of demineralizing agent at the surface of the bone.

b) It removes the gas envelope which surrounds the bone in non-agitated solutions and which presents a gas-bone interface (a diffusion barrier) rather than a liquid-bone interface.

c) It insures a more equal distribution of the salts removed from the bone through the solution, thus preventing a concentration in the immediate vicinity of the bone.

2. The effect of variation in temperature upon the rate of demineralization:

In the temperature experiments, temperatures of 25, 35, 45, and 55°C. were tested for increased rate of demineralization. Increase in temperature did produce an increase in rate of demineralization. Two factors probably are involved: (1) the factor of increased diffusion activity, and (2) tho factor of increased activity of the acid solution. The weight loss of the bone samples was increased more rapidly than was the weight of the mineral removed. From past experience, this indicates removal of materials other than mineral.

It was found that an optimal temperature range for

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demineralization lay between 25 and about 40°C. An increase beyond this range yielded a greater increase in total weight loss of the bone sample, but not a proportional increase in weight of mineral removed from the bone. The best results in terms of a total weight loss - weight of mineral removed ratio were encountered at 35°C.

Tests to determine the effect of acid solutions for hydrolysis and denaturation of proteins at increased temperatures have not been made; however, biochemical information available indicates that at temperatures above 40°C. many, if not all, of the protein complexes will suffer some change and that hydrolysis by the acid will be increased. The exact nature of the changes wrought by increased temperature upon the morphology and stainability of soft tissues associated with bone awaits investigation.

3. Effect of variation of the concentration of an acid upon rate of demineralization:

Experiments using various concentrations of hydrochloric acid have demonstrated that an efficient concentration range of acid exists for demineralization. Increases beyond about 2 molar concentration do not yield an increase in the rate of mineral removal commensurate with the increase in total weight loss in the sample. Concentrations of acid below 0.2 molar operate at a very slow rate and are impracticable because of the tremendously increased time factor. The optimal concentration of hydrochloric

acid has been found to be about 1 molar. Above this concentration efficiency of mineral removal drops when compared to total weight loss of the bone sample, while below it, efficiency decreases in terms of a slower rate of mineral removal.

4. The relative efficiency of various acids as demineralizers:

The acids commonly used in demineralization, as well as some chelating agents, have been tested for their relative efficiency. Two factors have been found to be important in determination of rate of demineralization: (1) the dissociation constant of the acid, and (2) the solubility of the calcium salt of the acid. The interplay of these two factors will determine, in part, the rate of demineralization. An acid with a high dissociation constant and a high solubility of the calcium salt will be a rapid demineralizer apparently due to the rapid breakdown of the bone salts and the rapid removal of the calcium salts. A low dissociation constant and solubility of the resultant calcium salt produces a slow demineralizer. However, it has been seen in experiments that a low dissociation constant can be compensated partially by a relatively high calcium salt solubility, and such an acid may prove to be as efficient an agent in demineralization as an acid with a higher dissociation constant but lower calcium salt solubility.

It has been found that some acids, which are excellent demineralizers according to rate, are relatively inefficient or

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wasteful in their action. It has not been determined whether this excess of acid is occupied in some un-wanted side effect such as hydrolysis or denaturation of protein. Comparative work on the soft tissues associated with bone may answer this question.

An efficient acid has been defined as one which uses a low equivalent of hydrogen to remove a high equivalent of mineral from bone. This does not indicate efficiency in terms of rate, but does specify a situation in which there should be minimal acid reactions with soft tissue components. These results, as do the other findings in the study, indicate that the most efficient acid in terms of hydrogen equivalents used, in rate of demineralization, possibly, in terms of side effects is hydrochloric acid.

Study of Soft Tissues:

Studies of blood and marrow smears have been reported previously (See Progress Report, September 15, 1953), and the conclusions are included in that Report.

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SUMMARY OF REPORT

December 31, 1953

The results of the study carried out during the past year on physical factors involved in the demineralization of bone has demonstrated the basic limitations imposed upon methods of removal of mineral from bone and has analyzed some of the factors that increase the rate of demineralization.

The basic limitations upon the rate of demineralization may be summarized as:

1. Surface area: This is the major limitation in demineralization because the rate of removal of bone salts is limited by the amount of surface available to the action of the demineralizing agent. The greater the surface area of bone exposed, the more rapid the rate of demineralization; the smaller the surface area per unit of bone mass, the slower the rate of demineralization under any given conditions.
2. Breakdown and removal of mineral from bone: This is the second limitation imposed upon that of surface area available. Several factors contribute and interrelate to produce this limitation:
   a) The diffusion distance: This determines the rate of diffusion of the acid into the bone and the rate of removal of calcium salts from the bone by imposing a diffusion gradient along which all

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materials must move. The greater the diffusion distance, the greater the time necessary for an acid or salt to traverse it, and the slower the rate of demineralization.

b) The dissociation constant of the demineralizing acid: The dissociation or amount of available hydrogen ion in an acid is a determinant of the rate of breakdown of the bone salts. The higher the ionization of the acid, the more rapid the rate of demineralization.

c) The solubility of the salts of the demineralizing acid: This is a determinant of the rate of removal of the salts from the bone. Relatively insoluble salts cannot diffuse rapidly even though being produced rapidly at the site of mineral breakdown.

d) The amount of liquid within the demineralized portion of the bone:

This is a factor which to a considerable extent controls the three enumerated above (Cf., a, b, c), because diffusion, ionization of the acid for breakdown of the bone mineral, and solubilizing of the calcium salt of the acid all demand liquid. Thus, the amount of liquid available within the bone matrix following the mineral removal from the region will be a determinant of demineralization.

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Factors that may increase rate of demineralization are as follows:

1. Concentration of acid: An optimum concentration of acid apparently is around 1 molar. Increase of the concentration beyond 2 molar does not correspondingly increase the rate of removal of mineral. Concentrations below 0.2 molar are so slow in removal of mineral as to be valueless.
2. Effect of temperature: Temperature increase beyond 40°C. does not yield a concomitant increase in the rate of demineralization. The unwanted side effects of damage to soft parts may increase at the higher temperatures. The optimum temperature for removal of mineral from bone proved to be about 35°C.
3. Agitation of the bone sample: Agitation of the bone sample during demineralization increases the rate about 25%.
4. Efficiency of the demineralizing agent: Of the demineralizing agents studied to date, the most desirable is hydrochloric acid which has a high dissociation constant, a high solubility of its calcium salts, a high rate of demineralization, and apparent lack of deleterious side effects.

Experiments on soft tissues:

Experiments with soft tissues have demonstrated that blood and bone marrow smears can be protected from acid concentrations up to 2 molar for as long as eighteen hours by fixation with a methyl alcohol - metallic iodine solution. Following acid treatment the morphology and stainability of these smears with

Wright's and Giemsa stains is unimpaired and yield results which are difficult to differentiate from normal slides made by the same technique.

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