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STUDIES OF BONE DEMINERALIZATION

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

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In the previous reports submitted to the AEC under Contract No. AT-(40-1)-1542 on Sept. 15, 1953, and Dec. 31, 1953, results of experimental studies were presented which demonstrated some of the basic physical limitations involved in the demineralization of bone. In addition, other data were presented which showed the effect of the variables of temperature, acid concentration, and agitation upon the basic limitations in demineralization. These various factors were summarized in the Progress Report of Dec. 31, 1953, as follows:

"The basic limitations upon the rate of demineralization:

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 materials must move. The greater the diffusion distance, the greater

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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."

From the experimental evidence the conclusion was drawn that while surface area will be the major limitation in the rate of

demineralization initially, it will be progressively less important as the demineralizing process continues because the surface area of the non-demineralized portion of the bone will continually decrease. In addition it was posited that the distance of diffusion for the demineralizing agent into the bone and the soluble salts out of the bone through demineralized matrix will increase. This, like surface area should bring about a decreasing rate of demineralization. In an effort to determine the effects of diffusion and surface area an experiment was devised to demonstrate the influence of each of these components in the same system.

I. Study of Diffusion:

As in previous studies (Report of Sept. 15, 1953), standard bone samples of human dried cortical femur were used for the experiment. A bone sample was given the shape of a bar and run in a solution of 0.5 molar hydrochloric acid for six hours. At the end of this period of time the demineralized matrix was removed, the surface area and weight again determined, and the bone sample run under the same conditions for a second six-hour period. During the total twelve-hour period of demineralization, the bone was weighed and X-ray pictures taken of both the front and profile views at half-hour intervals. The demineralizing solution of hydrochloric acid was changed at two-hour intervals. The depth of penetration into the bone sample by the demineralizing acid was measured and the surface areas calculated for each half-hour period. Calcium and phosphorus determinations were made and results of this study are presented in Table I.

The data on the weight and surface area of the original sample as well as the sample following removal of the matrix after

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six hours of demineralization are as follows:

	Weight	Surface Area
Original sample:	1.131 gm.	4.81 sq. cm.
After matrix removal:	0.144 gm.	2.75 sq. cm.

Results:

Examination of the data summarized in Table I shows that the penetration of acid into the bone was nearly equal for all external surfaces. Because of this, the undemineralized portion of the bone sample tends to maintain the form of the original bone; two differences in the regularity of penetration were observed however: (1) There was a slight difference in penetration of the ends and sides of the bone sample. This difference in penetration is believed to be the result of the orientation of the bone's structure to the solution. The bone sample was formed into a bar in such a manner that the Haversian canals were cut at right angles only at the ends of the bone sample while the sides of the sample were smoothed parallel to the Haversian systems. The Haversian canals of the Haversian systems will permit an easier entrance of a demineralizing solution as well as a larger diffusion area for removal of the mineral salts. Since the Haversian canals were open to the solution at the ends of the bone sample in this experiment, it would be expected that the penetration on the ends would be more advanced than that of the sides. Examination of Table I demonstrates this to be the case. Review of other experiments in which we have taken measurements of bone samples shows that demineralization always proceeds at a slightly greater rate upon those surfaces of the bone where the greater number of Haversian canals have been opened to the solution. (2) It was observed that while the undemineralized portion of the

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bone sample tends to maintain the shape of the original, the edges and corners gradually become rounded. This, we explain on the basis of the observation that two or more surfaces are presented to the action of the demineralizing agent, thus allowing more rapid removal in this region. This more rapid removal results in the rounding of the edges and corners of the bone sample. This rounding imposes a cumulative error upon our calculations of surface area and volume of demineralized bone. This error, of necessity disregarded, slightly decreases the surface area and increases the volume of demineralized bone. The calculations are, therefore, only an approximation.[1]

If the weight of mineral removed in milligrams is observed, it is noticed that the initial weight loss and weight of mineral removed is maximal during the first two-hour period; during the subsequent four hours there is continual diminution of mineral removed from the sample. In our previous reports, we have suggested that this initial loss is due to the greater surface area and that the subsequent lessening of the mineral removed is the result of diminishing surface area as well as a diffusion process.

Study of the measured penetration in millimeters into the bone on the end and the sides shows the penetration to be about 0.5 millimeter during the first two-hour period; during the subsequent four hours of demineralization the penetration was a total of 0.4 millimeter, which is less than the penetration during the first two-hour period. These data show that the more deeply the demineralizing

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1. The X-ray negatives were enlarged 15 diameters and measured to the nearest 0.1 mm with vernier calipers.

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agent penetrates into the matrix of the bone sample, the more slowly it penetrates per unit time. This decreasing rate of penetration per unit time correlates directly with the slowing of the demineralizing process.

Thus, the depth of penetration into the bone before removal of the matrix during the first two-hour period on the end of the sample was 0.54 mm. During the second two-hour period the increase in depth of penetration was 0.26 mm, and during the third two-hour period was 0.14 mm. This decreasing increment of penetration is approximately 50% per unit time. The variations from 50% are within the limits of error of the measurement taken on X-ray film.

Additional evidence of the behavior of the decreasing increment of penetration per unit time is provided in the second part of the experiment. Here, the demineralized matrix was removed, the sample again run for six hours, and the increments of penetration measured. The increments of penetration are almost identical to those of the first six-hour period (Cf. Table I). The weight loss, however, was only a fraction of that of the previous six-hour run. It would appear that this decreasing increment of penetration is related neither to surface area nor to the weight of the sample since both of these factors were altered when the demineralized matrix was removed.

A decreasing increment of the weight of mineral removed per unit time also exists (Cf. Table I). During the first two-hour interval, before removal of the demineralized matrix, 147 mg of mineral were removed; during the second two-hour period 57 mg; and during the third 40 mg. Following removal of the matrix the mineral removed also showed a proportional decreasing increment similar to

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that of the first period (Cf. Table I). Unlike the distance of penetration, however, the amount of mineral removed during each two-hour interval for the two six-hour periods differs. This would be expected from the results of previous experiments in which it has been shown that the amount of mineral removed per unit time is directly related to the surface area exposed to the action of the demineralizing agent as well as the total amount of bone sample present (See Progress Report, Sept. 15, 1953, Surface Area Experiments).

It is significant that upon removal of the demineralized matrix at the end of the first six-hour period the rate of penetration immediately returns to that of the initial value which was 0.54 mm. Comparison of the first two-hour periods of both runs shows the penetration to be identical, in the second two-hour period to vary in one dimension by 0.002 mm; in the third two-hour period to vary in one dimension by 0.004 mm. These figures are well within the limits of error of measurement from the X-ray film. In each case the rate of penetration into the bone was reduced by approximately 50% per two-hour time period.

Calculations of the volume of bone demineralized show that at the end of the first six-hour period the bone sample had a demineralized volume of 343.8 cmm, or about 57% of the total volume of the bone was demineralized. At the end of the second six-hour period the bone sample had a demineralized volume of 183.96 cmm or about 76% of the total volume was demineralized. If these volumes of bone are divided by the total amount of mineral removed during the demineralization, it is 4.06 mg/cmm for the first bone sample and 4.18 mg/cmm for the second. This is considered to be identity. Thus, the amount of mineral removed per unit volume is the same even though the

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mineral and weight loss are quite different.

We now feel that it is possible to draw our major conclusions as to the basic limiting factors in a given system of demineralization. (1) It has been demonstrated by this experiment that under a given set of controlled conditions penetration into a standard dense cortical bone of the demineralizing agent will take place at a decreasing rate. This rate is independent of weight of the bone sample as well as surface area of the bone sample. (2) The amount of mineral removed per unit time or per unit of penetration is directly correlated with the surface area available for mineral breakdown. (3) As this surface area is continually decreasing, the amount of mineral removed will also continually decrease. (4) Since the penetration into the bone involves a decreasing rate, the relative bone mass (distance from the surface to the center of the bone piece) will be the determinant of the total amount of time required for demineralization.

It is recognized that these experiments have been made under ideal conditions. Nevertheless, they are applicable to conditions in irregular bone pieces and bones composed of both compact and cancellous bone. It is more difficult to calculate and predict the exact behavior of such bone pieces, but a rough estimate of demineralization time may be derived from extrapolation of these principles to a given bone piece.

II. <u>Experiments with various types of bone</u>.

These experiments were made to determine the rate of demineralization, and variability in weight loss and mineral removal in various types of bones. Further, it was hoped that these experiments would reveal a pattern of demineralization for var-

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ious kinds of bones if such a pattern existed.

Method: Five bones were used from adult male Wistar rats: tibio-fibula, innominate, femur, humerus, and calcaneus. These bones were fixed in 10% formalin for 24 hours and after fixation were cleaned to remove the soft tissues and periosteum. The bones were placed in 100 ml of 1 molar hydrochloric acid and placed upon a 60 rpm roller agitator at 25°C. X-ray pictures and weighings were made at one-hour intervals to determine the degree of demineralization. Calcium and phosphorus determinations were made on the demineralizing solutions at the end of demineralization.

Results: At the end of four hours, study of the X-ray pictures showed each bone to have a small undemineralized area located in the center of that portion of the bone farthest from any external surface. In the calcaneus, this was a thin undemineralized bar through the center of bone; in the case of the innominate bone the undemineralized portion was located about the acetabulum. In the humerus the undemineralized portion was in the head at the thickest portion. The tibio-fibula showed undemineralized parts both in the shaft as well as in the ends of the bone. After five hours of demineralization the picture was only altered in detail, the same areas still being undemineralized. Table II is an average of two experiments, each with a similar set of bones, which shows that the weight loss of the individual samples was about the same. The amount of mineral removed from each bone was within 1 mg. % for calcium and phosphorus for the same bone samples. The tibio-fibula and the innominate both lost about 38% of their weight; the humerus lost about 42%; and the calcaneus showed the higher percentage of weight loss. In the case of the tibio-fibula and the

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innominate bones, the amount of mineral per unit weight loss was 75% while in the humerus it was 71% and in the calcaneus 70% of the total weight loss.

In every case, the conclusions reached in the studies on standard bone were confirmed: that part of the bone which was farthest from any external surface was invariably the last to demineralize while the thin portions were always quickly demineralized. The size of the bone was not of significance in the amount of time necessary for demineralization, this being controlled by the diffusion distance (i.e., demineralization of the calcaneus, weight c. 72 mg, took as long as the humerus and tibio-fibula, weight c. 600 mg).

III. Experiments to determine the relative efficiency of various acids on demineralization of rat femora.

Wistar rat femora were fixed in 10% formalin and the soft tissues and periosteum removed. These bones were placed in 100 ml of 1 molar or 0.5 molar concentrations of the following acids: hydrobromic, hydrochloric, trichloroacetic, phosphoric, acetic, and nitric. The bones were agitated on a 60 rpm roller agitator at 25°C. X-ray pictures and weighings were made at regular intervals, and calcium and phosphorus determinations were made on the total solution at the end of the run.

Results: The relative rank of these acids was identical to that previously seen (Cf. Report of December 31, 1953). Hydrochloric, hydrobromic and nitric acids demineralized the bones in 6 hours. Trichloroacetic acid came later at 12 hours, phosphoric acid shortly thereafter, and finally the slowest was acetic acid, not being completely demineralized after 24 hours demineralization.

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IV. Experiments with ion exchange resins.

Standard rat femora were prepared in the method outlined above and placed in solutions of 1 molar and 0.5 molar hydrochloric acid, each solution containing 25 gm wet weight of a different resin. The resins were Rohm and Haas IRC-50, IR-120, and IR-112, and Winthrop-Stearns Win-3000. The bones and resins were agitated on a 60 rpm roller agitator at 25°C. The bones were weighed and X-ray pictures made every hour. Calcium and phosphorus were determined in the total solution. The acids were standardized prior to the run and again titrated at the end of the run. These data are presented in Table III.

Preliminary Results: These are results of experiments in progress and further data are being collected which may change our present conclusions. In general, the results of these experiments show that while resins plus acids do not appreciably increase the demineralization rate, the solutions do appear to act more efficiently.

The mineral determinations for the resin solutions are not informative due to the binding capacity of the resins which remove the mineral from the solution. Calcium was the primary mineral removed from the solution, phosphorus being taken out of solution only to a limited extent. The most efficient remover of calcium from the system was IR-120, then Win-3000, IR-112, and finally IRC-50 which accepted the least amount of calcium ion.

Comparison of the data presented in Table III demonstrates almost no difference in the activity of 0.5 and 1.0 molar hydrochloric acid plus various resins, and only a small difference between 0.5 and 1.0 molar hydrochloric acid alone. A major

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difference is found when the resin-acid solutions are compared with the results of acid solutions alone. Analysis of the X-ray negatives shows no difference in amount of demineralization between the acid alone and the resins plus acid at a given time. Thus, demineralization appears to progress at the same rate with and without resins as viewed in X-ray negatives. Weight loss figures, however, would seem to indicate that bones in the resin solutions are not demineralizing as rapidly because the resulting percent weight loss often is as much as 5 percent points less than those in acid alone (Cf. Table III). It appears from this comparison that this difference may be due to something else being removed from the bone beside mineral in the case of the acid alone.

If terminal figures after complete demineralization in a 0.5 molar solution of acid are compared, the percentage weight loss for the femora in the various solutions is:

HCl	39.1%
Win-3000 + HCl	38.6%
IRC-50 + HCl	37.1%
IR-112 + HCl	36.5%
IR-120 + HCl	35.2%

It is seen that a differential of 4% has developed during the demineralization period, yet all the bones are completely demineralized. Correlations may be drawn on this evidence. The mineral content of the rat femur is about 30 to 31% of the total weight of the bone; the carbon dioxide content is about 2-3% of the total weight of the bone. Thus, the expected weight loss of the bone during demineralization may be expected to be from 32 to 35% of the total weight of the bone at complete demineralization. In the table above, the hydrochloric acid alone removes 39% of the total bone weight in complete demineralization, or from 4 to 7% more weight

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than can be accounted for by the mineral and carbon dioxide present in the bone. IR-120, on the other hand, removes only 35% of the bone weight to accomplish complete demineralization, or from 0 to 3% more than can be expected as salt product from demineralization.

It would appear that removal of mineral in the resinous system is just as efficient as that in the hydrochloric acid alone, but that unwanted side effects in removal of other materials does not occur to as great an extent in at least two of these systems (IR-112 and 120). Experiments at present in progress are expected to yield more precise information as to the possible significance of these present findings.

The standardization of the acid solution following demineralization in these systems shows that IRC-50 has the same molarity after demineralization as before, but all other solutions show a slight reduction in molarity.

In addition to the above experiments, chemical studies have been conducted on the various resins. These studies have provided a base line of exchange capacity for calcium, phosphorus, and hydrogen in the calculations of the optimum amount of resin.

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XV. Studies of tissue preservation and staining after acid action.

To test the effects of acids upon tissues following various fixation in terms of preservation of morphologic details, shrinkage, staining, and tissue friability, a series of experiments similar to those previously reported for blood and bone marrow were conducted (Progress Report, Sept. 15, 1953). Rats served as the source of tissue for these studies.

Method: The tissues were either perfused with or submerged in the fixing solution. Perfusion of the animal was accomplished by inserting a cannula into the dorsal aorta and washing the vascular system with Ringer's solution which was followed by the perfusing liquid. The skin was removed from the hind legs of the animal and the muscles of the legs transected to make sections approximately 5 mm thick. The entire leg with attached muscle was then submerged in the fixing solution for at least 24 hours. Following fixation, muscle pieces 5 mm thick and usually a centimeter in its other dimensions were cut from the legs, washed in distilled water, and placed in polyethylene containers with 100 ml of a 2 molar concentration of acid. The polyethylene containers were run on a 60 rpm roller agitator at 25°C. for 24 or 48 hours. The pieces were washed in water, dehydrated in ethyl alcohols and embedded in paraffin. Paraffin sections were cut and stained with haematoxylin and eosin or with a one-step trichrome devised in this laboratory. The slides were given code labels and the sections analyzed without knowledge of what procedure had been applied. The evaluations were made and then compared to a key sheet. The slides were evaluated according to the following points:

1. Preservation of morphologic detail.

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2. Stainability and color balance.

3. Shrinkage.

4. Friability of the tissue.

Four fixatives were used: 10% formalin, Bouin's, iodine-methyl alcohol, and methyl alcohol. Tissues were fixed in these solutions both with and without perfusion. The tissues fixed in these fluids were placed in the following aqueous solutions: citric, hydrobromic, nitric, trichloroacetic, phosphoric, hydrochloric, acetic and formic acids, and saturated sodium versenate. Fixed tissues not treated with acid solutions served as controls.

Results:

The quality of the morphology of the tissues after acid action is the resultant of several factors:

1. The general effectiveness of the fixative and its result with the stains used.
2. The effectiveness of the staining technique in demonstrating the morphology.
3. The destruction of morphology resulting from the action of the acid solution.

As side issues, it is evident that an acid which destroys all the connective tissue will reduce the tissue to a mass of cells, and while the morphology of these cells may be perfect, the result defeats the purpose of studying the relationships of structures and cells *in situ*. Also, the resulting tissue will not be better than the fixation and staining regardless of the effect of the acid.

Fixation:

Bouin's fixation: Following this fixation, good cellular morphology was maintained in most acid solutions.

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Bouin's did not give adequate protection against the stronger mineral acids such as nitric and hydrobromic. The fixation of the connective tissue was generally poor and these tissues were not maintained in even dilute concentrations of weak acids unless the water soluble proteins were further fixed by alcohol or some other agent. The nuclei usually lacked brilliance and clarity following this fixation and even more variable results in nuclear staining were obtained following the acid action. In general, the nuclei were poorly stained unless some additional fixative was employed.

10% Formalin: Good protection and staining results were obtained after fixation in this solution. The fixative with acid action did make the tissue more easily "cooked" during paraffin embedding. Nuclei were generally good, but some poor stains were obtained after hydrolysis in the strong mineral acids.

Iodine-methyl alcohol: This fixative in our opinion gave the finest results because the tissues did not appear to be fixed at all but had the appearance of fresh tissue. It is necessary to point out, however, that the results with this fixative might be considered very poor if the typical fixation picture is expected. By way of demonstration, muscle fibers fixed with formalin or Bouin's are usually polygonal in shape in cross section; with methyl alcohol-iodine, the fibers are round, closely packed, with brilliant and numerous nuclei. Shrinkage is minimal. Fresh muscle fibers in

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cross section without fixation are also round, closely packed, and with vital stains show numerous nuclei. Thus, while in our own estimate, this fixation gives the most satisfactory result, it would not be considered "normal" because of the changes which are usually expected in the processing of the tissues.

With this fixative, disasterous results were obtained with nitric and hydrobromic acids. The tissues were completely digested by these acids. Good results were obtained in the other acid solutions however, and little or no morphologic alteration could be noticed when compared with the non-acid control blocks.

Methyl alcohol: Results with this fixative were always poor.

Several fixatives were not considered because of the difficulties which they cause in the demineralization method. Bichromate or mercuric salts, which are excellent fixatives, were not used because of the problems of common ions in solution; the metallic ions are removed at the expense of calcium ions.

Staining:

The staining characteristics of the tissues were governed by the acid used. With haematoxylin and eosin, it was frequently difficult to obtain nuclear staining following use of most of the acids. The weak organic acids such as acetic and citric give good results, but the others produce poor haematoxylin stains.

Different parts of the tissue stain differently in the stronger acids; the periphery stains poorly or not at all while the interior of the block may give good results. Counter stains are

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usually good, but use of the one-step trichrome shows that pH is apparently a factor which must be controlled.

In general, staining after nitric and hydrobromic acids is most difficult if not impossible. Hydrochloric and formic acids give variable results, from excellent to poor depending more upon the type fixative than upon the acid. Trichloroacetic acid shows a considerable variation, but is usually less satisfactory than hydrochloric and formic. The weak organic acids and the sodium salt of versenate give good staining results for almost all tissues.

To summarize the results of these studies, it has been found:

1. Strong acids such as nitric, hydrobromic, and hydrochloric acids as well as trichloroacetic tend to make the tissues more friable and more easily cooked in the embedding process. The tissues frequently shred and shatter during sectioning and show gross morphologic changes. Shrinkage is maximal with these acids after formalin or Bouin's fixation.
2. The nuclei are difficult to stain after treatment with most of the acids. This has been the major staining problem.
3. (a) Fixation in iodine-methyl alcohol gives the best results if nitric or hydrobromic acids are not used.
   (b) Fixation in Bouin's produces poor nuclear staining and some destruction of connective tissue by the acid solutions. The collagenous fibers are usually swollen after acid solutions with this fixative. If the Bouin's is followed by alcohol, better preservation of the con-

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nective tissue is obtained.

(c) Formalin fixation gives good protection to the tissues but makes them more friable.

4. The connective tissues are destroyed by the strong acids such as nitric and hydrobromic. Good results were obtained with hydrochloric acid.

Thus, in conclusion, the major problem is to effect good staining after acid action because the other factors can be controlled by proper attention to the mechanical details of slide making.

Because of the difficulty in staining the tissues after the action of acid, it was decided to undertake a series of experimental studies to determine some of the factors which govern the acceptance of the dye by the tissue in order that corrective procedures might be applied. The work was outlined to investigate the following points:

1. The effect of the changes in reaction pH.
2. The effects of dye concentrations.
3. The effects of mordants.
4. The effects of acid hydrolysis and hydrolysis of other chemical agents.

VI. Study of the pH of tissues and the changes in pH.

Method: Sections were stained with neutral red and the slide flooded with either an acid or alkaline solution of known concentration. The slide was observed and the color changes and the time necessary for the change of the pH reaction of neutral red in the slides noted.

Results: In this experiment, it was found that it requires 15 to 20 minutes for an acid solution of 0.01 molar concentration to

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shift the pH one color point. Increasing the acid or alkali concentration does not markedly affect this rate of change, although in concentrations of 1 molar, the rate may be as much as 50% faster.

If a tissue is made strongly acid to neutral red, an alkali also requires a considerable period of time to shift the pH to an alkaline indication. In general, it has been found that it requires from 30 minutes to one hour to make a substantial change in the pH reading of the tissue, if it previously has been exposed to either an acid or alkaline solution.

VII. Dye displacement.

Method: Tissues were stained with one of the following dyes: aniline blue, acid fuchsin, orange G, eosin, azo carmine B, or fast green. The tissues were then washed in water and placed in another of the above series of dyes.

Results: In each case, using all the possible permutations of the above system, the first dye was displaced by a second dye. In some experiments, as many as five dyes were displaced from the tissue, with total staining of each successive dye. The tissues were then placed in the first dye and the original color again obtained.

VIII. Reaction pH.

Method: Slides from the same block were adjusted to pH 1 to 11 by placing them in a 0.9% NaCl solution buffered to the exact pH with HCl-NaOH. The slides were allowed to equilibrate for 1 hour in the various pH solutions before staining. A set of slides from pH 1 to 11 were stained in a 1% concentration of the following dyes for 1 minute: aniline blue, Wasserblau, acid fuchsin, orange G, fast green (FCF), and haematoxylin. The haematoxylin was reduced in

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alkali.

The slides were washed in water, dehydrated in ethyl alcohols and mounted.

Results: Variations in the staining were obtained. With haematoxylin, the dye stained over the entire range but the lower and higher pH values gave more brilliant results. Orange G did not stain beyond pH 5. Fast green faded progressively until no stains could be found above pH 10 although the dye did not stain maximally beyond pH 5. Aniline blue alone does not stain beyond pH 7; Wasserblau stains throughout the entire pH range but is at its weakest in the middle pH range from 6 to 8. Acid fuchsin stains throughout the entire range but is weaker on the alkaline side.

IX. Reaction pH in polychrome systems:

Method: Tissues of the same block of tissue were adjusted to pH 1 to 11 in 0.9% NaCl buffer with HCl-NaOH solution and stained with the one step trichrome for 1 minute.

Results: With the one step trichrome, it was found that at the lower values of pH 1-5 the best differential results could be obtained. As the pH approached 10 the orange G, most of the fast green, and almost all of the acid fuchsin were eliminated and the tissues stained only with the primary blue or at times a blue-green color.

X. Effect of Acid Hydrolysis.

Method: Tissues fixed in 10% formalin or Bouin's were hydrolyzed in 1 molar hydrochloric acid solution for 1 hour at 56°C.; the reaction pH was adjusted to pH 3 with 0.9% NaCl buffer with HCl-NaOH solution and stained with haematoxylin and eosin or with the one step trichrome. Tissues from Experiment V which had been

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agitated in 2 molar hydrochloric acid solution at 25°C. for 48 hours were also used. These tissues were adjusted to pH 1 to 11 and stained in haematoxylin and eosin and in the one step trichrome.

Results: With haematoxylin, the tissues stain best after adjustment to an alkaline reaction pH, that is, from pH 8 to 11. In the acid range, the nuclei are very difficult to stain and the cellular outlines are not demonstrated in the blood vessels or the connective tissue. Adjustment to about pH 7 gave a more satisfactory staining with the one step trichrome. Thus, following acid hydrolysis, the reaction for optimal staining with haematoxylin is pH 9 to 11, and for one step trichrome about pH 5 to 8.

XI. The effect of oxidizing and hydrolyzing agents.

Method: Tissues were oxidized in 3% potassium permanganate with and without subsequent wash with 1% potassium meta bisulfite. Other tissues were hydrolyzed for 1 hour in 1% periodic acid with and without a 1% potassium meta bisulfite wash. The tissues were adjusted for pH in buffer solutions and stained with either haematoxylin and eosin or with one step trichrome.

Results: The results after strong oxidation with potassium permanganate showed that only the fuchsinophilic material remained; a slight, but weak staining with aniline blue was obtained at times. The alkaline adjustment of pH after oxidation did not show any marked improvement. The staining with haematoxylin was poor throughout the series.

The staining reaction following hydrolysis with periodic acid showed an increased brilliance regardless of the adjusted pH, but best results were obtained in the pH range of 3-5.

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XII. Use of mordant salts.

Method: Tissues were hydrolyzed in hydrochloric acid, periodic acid, or run as untreated controls. These tissues were then placed in potassium bichromate solution, Zenker's sublimate, mercuric bichloride, gold, silver, osmium, barium, calcium, and ammonium salt solutions for periods of 1, 2, 3, and 4 hours before adjusted to pH 3 and stained with the one step trichrome or with haematoxylin and eosin.

Results: Some of the mordant solutions restored the stainability of the tissue with haematoxylin but best results were obtained with mercuric bichloride. This mordant appears to completely restore the nuclear stain after acid hydrolysis.

It was found that the one step trichrome showed considerable variation in staining depending upon the length of time the tissue was left in the mordant solution. The longer the tissue remained in bichromate solution, the more the tissues differentially accepted the acid fuchsin of the trichrome solution. Muscle moved from an orange red at 0 hours to a brilliant scarlet after 4 hours in potassium bichromate. Secretion granules were differentiated as a bright red after the mordant while they were dark purple or unstained in the control solution.

Discussion of Experiments:

In routine histologic techniques, the only material which remains to be stained after fixation, dehydration and sectioning is the protein structures. The carbohydrates and fats are usually dissolved out either in the aqueous or the organic solutions used in preparation. Thus, it is probable that the staining which takes place must be a reaction between the dyes and the protein stroma

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remaining after the histologic processing.

In order for a colored substance to dye or hold fast to a tissue it must have some anchoring group or groups. These anchor groups or auxochromes are of two general types, the acid and the basic, which are:

Basic	Acidic
$R-NH_2$	$R-SO_3H$
R-NH	R-COOH
R-N-R	R-OH

Dyes which have basic auxochrome groups ($R-NH_2$, R-NH, R-N-R) will form salts with acid radicals in the tissue and these are basic dyes; the acidic group will combine with the basic groups in the tissue to form dye salts and are the acid dyes.

The dyes may react in two ways, either directly or through another chemical compound or mordant. The direct dyes probably react primarily with the amino or carboxyl groups of the protein molecules but some direct dyes are also selective and form compounds with a specific dye nucleus within the tissue (e.g., SO_3). With direct dyes, the concentration gradients and dissociation of both the dye and the protein substrate are important conditioning factors for staining.

Mordant dyes are unable to act without some adjunctive group or mordant. These adjunctive groups must be combined with the protein groups to form a chemical bridge between it and the dye. The most useful mordant ions are those of chromium, iron, and mercury. Frequently, it is possible to substitute an auxochrome group into a molecule by use of a mordant.

In the trichrome system which we use for staining tissue

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elements, all the dyes are sulfonated acid dyes. They are usually sodium salts of sulfonic acid. These stains are dye salts and act from acid solution. The staining reaction which takes place demands a dye salt since the free color acid is neither easily soluble nor easily accepted into the substrate molecule.

In the experimental results (Cf. VI) a tissue stained neutral red which is placed in either acid or alkaline solution takes a considerable time to make minor changes in pH of the tissue. This is a crude indication of the buffering capacity of the protein stroma and its resistance to pH change. Thus, when the buffer effect of a tissue has been shifted with either acid or alkali, it will require a considerable time in a dye solution to readjust to the proper staining pH. Because differential stains depend upon the more rapid staining of one element over all others, short staining periods are necessary; with the increase of time necessary for readjustment of the buffer effect, all the elements stain and the differential effect is lost.

In the experiment on dye displacement (Cf. Exp. VII) it was found that when a tissue is moved from one sulfonated dye to another, dye II will completely replace dye I. From this, it may be interpreted that the dye or its chromophore group has little or no binding capacity. With such a weak bond, the dye in greater concentration will stain the tissue and displace the more dilute dye.

If additive protein compounds are made with metals, such as gold, silver, or osmium (Cf. Exp. XII), sulfonated dyes will not stain until these salts, both reduced and nonreduced, are removed. These metals usually combine with the carboxylic or hydroxy groups. Following the ion removal, the dyes will again stain the tissue but

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not so well as in the control slide.

Dyes form lakes or insoluble combination products with protein structures only within specific pH ranges. When a dye stains a protein, the intensity of staining is an index of the amount of dye-protein compound formed. The intensity of stain will be dependent, then, upon the concentration of both the dye and the protein ions in the reacting system. The concentration of the dye and the dye ions is a function of: (1) the amount of dye present, (2) the dissociation constant, and (3) the pH of the solution. The dissociation of the protein ions will be determined by the ionic strength of the solution, the pH, and the amount of ionizable protein present.

For the dye to remain in the tissue, the dye-protein complex must precipitate. Further, the amount of precipitate will follow the mass action law and be determined by the ionization and concentration of the dye or protein ions. The point of maximal precipitation of dye-protein complex will be the point of least ionization, and thus, the dye-protein complex has an iso-electric point. This iso-electric point may or may not differ from the iso-electric point of the protein or the pH range of insolubility of the dye. From this it has been concluded that there is a <u>reaction pH</u> or that pH at which the maximal reaction between the dye ion and the protein will occur and the dye-protein complex precipitate. At pH points other than the reaction pH, the dissociation of the dye-protein complex will shift the direction of the equilibrium from precipitation towards solubility of the components.

This concept was tested by the experiment in which the pH was adjusted to a pH 1 to 11 and then the tissue stained with the various dyes (Cf. VIII). The pH of the dye solution was optimal for

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its solubility, but when the adjusted pH of the buffer systems in the tissue exceeded pH 5-6, the dissociation of the dye-protein compound is increased and the equilibrium is shifted towards the solubility side. No staining results. Thus, orange G did not stain above pH 4, and fast green above pH 5. A strong acid dye in high concentrations can compensate for the buffer capacity of the tissue. This was shown by the difference between aniline blue and aniline blue-acid (Wasserblau) (Cf. Exp. VII).

Color intensity was found to vary upon manipulations of any one of several factors. Color intensity can be varied by the adjusted pH of the tissue or the pH of the staining solution. Color intensity can be varied by the concentration of the dye; the high dye concentration suppresses the dissociation of the dye-protein complex to shift the reaction to precipitation. Finally, the ionic strength and molarity of the buffer used to adjust the tissue pH before staining will influence the color intensity: high concentrations and ionic strengths will suppress staining while low concentrations of buffer will increase the color intensity.

The intensity of the dye compounds in the complex trichrome system (Cf. Exp. IX) seems to be directly related to the pH of the tissue components. If the tissue was adjusted to approximately pH 3 the best color balance was obtained. As the tissue was adjusted to a more alkaline reaction, only the strongly acid dyes seemed to combine with the tissue elements.

After hydrolysis with acid solutions (Cf. Exp. X), it was necessary to adjust to a more alkaline pH level for adequate staining.

Therefore, it has been learned that: (1) staining

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intensity of a dye-protein compound can be controlled, and (2) that dilute solutions of buffer are better than more concentrated solutions, and (3) that optimal staining pH does not occur at the iso-electric point of the protein itself but is the iso-electric point of the dye-protein compound.

Results from this study, while showing staining improvements, were not entirely satisfactory in the hydrolyzed tissues because even after pH adjusting they were not as brilliant as desired. The mordant series of experiments were run as a possible answer (Cf. Exp. XII). Haematoxylin in the unmordanted system did not stain effectively, but after soaking the slide in mercuric bichloride the nuclear chromatin was stained an intense blue. The intensity of the haematoxylin stain was also improved after soaking the slide in potassium dichromate. It was found with the one step trichrome that the longer exposure to the dichromate salt increased staining intensity with acid fuchsin and depressed staining with the aniline blue.

Tissues were oxidized with potassium permanganate to determine whether this type of rupture was comparable to the molecular hydrolysis which occurs with acid (Cf. Exp. XII). The action of potassium permanganate gave consistantly poor staining results. Hydrolysis with periodic acid, however, increased the staining brilliance. Oxidation of protein by potassium permanganate will release ketone bonds as the end product; hydrolysis of some proteins with hydrochloric acid will release aldehyde groups. Hydrolysis with periodic acid will release adjacent hydroxy groups. Both permanganate and hydrochloric acid produce poor staining results and it is tentatively proposed that ketone and aldehyde

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groups are not responsible for trichrome staining. The staining of the tissues with trichrome would appear to be due, at least in part, to the intactness of the hydroxyl groups in the tissue.

To summarize, it has been found:

(1) Adjustment of the reaction pH can improve staining after acid hydrolysis. This conclusion had been tentatively reached previously in the study of blood cells and acid action (see report, Sept. 15, 1953).

(2) The reaction of sulfonated dye salts with proteins is believed to be dependent in part upon the intactness of the hydroxyl groups of the tissues. Alteration of the hydroxyl group by substitution, hydrolysis, or oxidation produce poor staining. Combination of the hydroxyl groups in the tissue with such salts as ammonia, potassium, calcium, or barium will remove the stain from the tissue.

(3) The dye-protein complex will precipitate only in specific pH ranges and these points are the iso-electric points of the dye-protein complex. Variations from this iso-electric point allow~~s~~ dissociation of the complex and the solubility of the dye-protein complex to aqueous solution. This solubility allows removal of the stain from the tissue.

(4) Mordants will form a dye-protein bridge after acid hydrolysis. Excellent results have been obtained by using mordants and controlling the reaction pH following acid hydrolysis. Mercuric bichloride mordant is the best means of reconstituting the stainability of

the nucleus with haematoxylin following acid hydrolysis.

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TABLE I

Data showing Wt. Loss, Wt. of Mineral Removed, Calculated Surface Area, and Depth of Penetration at two-hour intervals as described in Experiment No. 1.

Time (hours)	Wt. Loss (mg)	Wt. Mineral Removed (mg)		Surface Area (sq. cm.) (Calculated)	Penetration (mm)	
		Ca	P		End	Side
Before Matrix Removal:						
0				4.81		
2	160	106	41	3.74	0.54	0.50
4	227	41	16	3.31	0.80	0.70
6	273	29	11	2.97	0.94	0.86
Totals		176	68			
After Matrix Removal:						
0				2.87		
2	94	49	22	2.00	0.54	0.50
4	127	22	8	1.59	0.80	0.72
6	148	16	5	1.35	0.98	0.86
Totals		87	35			

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TABLE II

Data showing Average Percent Wt. Loss and Mineral Removed as Percent of Total Bone Wt. in rat bones as described in Experiment No. 2.

Time	Bone	Percent Wt. Loss	Mineral: Total Mineral as % of total bone wt.	Mineral: Ca as % of total wt.	Mineral: P as % of total wt.
2 hours	Humerus	31.9			
	Innominate	31.5			
	Tibio-fibula	28.2			
	Calcaneus	32.0			
4 hours	Humerus	39.2			
	Innominate	36.0			
	Tibio-fibula	35.0			
	Calcaneus	42.0			
5 hours	Humerus	42.0	29.8	20.8	9.0
	Innominate	38.1	28.0	19.9	8.1
	Tibio-fibula	38.0	29.1	20.7	8.4
	Calcaneus	45.0	30.1	21.8	9.3

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TABLE III

Data showing Percent Wt. Loss, Wt. of Mineral Removed, and Molarity of Acid Solution in demineralization of rat femora in various resins as described in Experiment No. 4.

Time		% Wt. Loss 0.5 M HCl	% Wt. Loss 1.0 M HCl
1 hour	IRC-50	21.4	21.6
	IR-112	20.2	22.4
	IR-120	19.5	21.3
	Win-3000	21.7	22.5
	Control	20.4	25.0
2 hours	IRC-50	29.0	31.0
	IR-112	27.4	29.8
	IR-120	26.0	28.9
	Win-3000	28.6	29.6
	Control	30.6	33.1
4 hours	IRC-50	35.5	35.2
	IR-112	35.0	36.1
	IR-120	33.7	33.9
	Win-3000	37.9	34.7
	Control	36.0	38.0
5 hours	IRC-50	35.5	35.4
	IR-112	35.0	36.8
	IR-120	33.7	34.3
	Win-3000	37.9	35.3
	Control	--	39.0
7 hours	IRC-50	37.1	--
	IR-112	36.5	--
	IR-120	35.2	--
	Win-3000	38.6	--
	Control	37.4	38.4

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TABLE III, contd.

Mineral Removed After Complete Demineralization

	Ca		P	
	0.5 M HCl	1.0 M HCl	0.5 M HCl	1.0 M HCl
IRC-50	138.78	146.08	57.78	64.43
IR-112	28.30	74.18	55.68	64.83
IR-120	10.23	28.99	62.10	67.57
Win-3000	11.14	45.65	60.80	61.60
Control	134.21	161.60	69.77	69.08

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Atomic Energy Commission Contract No. AT-(40-1)-1542

EXPENDITURES AND BALANCES*

	RECEIVED	DISBURSED	BALANCES
Salaries:	$12,100.00	$2,256.44	$9,843.56
Travel:	$ 250.00	$ 219.05	30.95
Equipment:	$ 1,575.00	$1,945.29	$370.29 (overspent)
Supplies:	$ 3,650.00	$1,114.27	$2,535.73
	$17,575.00	$5,535.05	$12,039.95 TOTALS

* Balances received from Business Office of the Medical College of South Carolina, as of August 31, 1954.

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Summary of the Progress Report

(Atomic Energy Commission Contract No. AT-(40-1)-1542)

September 15, 1954

The results of the studies carried on since the last report on December 31, 1953, have shown:

1. A diffusion process exists in the demineralization of bone. This diffusion process is one of the major limitations in demineralization because the rate is determined by the diffusion distance into the bone sample. This is independent of the weight of the bone sample as well as the surface area of the bone sample.
2. In experiments with irregular bones, it has been shown that the diffusion distance is the major determinant of the time necessary for demineralization. In every instance, that part of the bone which was the greatest distance from the surface was the last to demineralize.
3. Studies of the relative efficiency of demineralization on rat bones has been shown to be the same as previously reported for standard bone samples. (See Progress Report, December 31, 1953.)
4. Preliminary studies using ion exchange resins in the demineralizing system show that resins appear to give protection to the bone sample. The weight loss of the bone sample, which is an indication of the amount of material removed during demineralization, is less in systems of resin plus acid than in acid solutions alone at complete demineralization.
5. Studies of tissue preservation and staining have shown that

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the major problem is to effect good staining after acid action. All other factors can be controlled by proper attention to the mechanical details of slide making.

6. Studies of staining have revealed that control of pH of the tissue and use of mordant salts will reconstitute the staining characteristics of the tissues after acid hydrolysis.

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