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STUDIES ON DEMINERALIZATION OF BONE *

IV. Evaluation of Morphology and Staining Characteristics of Tissues after Demineralization

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The present work is the evaluation of the morphology and staining characteristics of the tissues after the various methods of demineralization. This study has also analysed the effectiveness of fixatives in preventing tissue destruction by acid hydrolysis, the staining reaction of cells and tissues, and various methods of correcting poor staining reactions after immersion in acid.

To make preparations of bone with associated soft tissues which 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 bone salts to soluble salts which can be removed from the tissue. Acid solutions are the only effective method yet found to accomplish this change.

The acid solution frequently alters the morphology or staining reaction of the tissues associated with the bone, and because of this, many attempts have been made to modify acid demineralizing solutions to prevent this unwanted alteration. These methods may be grouped into four general categories:

1041163

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 by Busch[2]; 50% hydrochloric 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 diffusion gradient out of bone; these systems are to protect the tissue by shortening the time necessary 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 Birge and Imhoff[1]).

In a series of papers, Morris and Benton[9, 10, 11] have analysed the relative efficiency of the various methods of demineralization under standard conditions. The criteria for a good demineralizing method were defined as rapidity of demineralization and a low amount of protein hydrolysis as demonstrated by the quantity of nitrogen in the demineralizing solutions after demineralization.

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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 concentrations of fixatives and acids upon the preservation of cytological detail and staining properties of blood cell smears.

Smears were prepared from human and rat peripheral blood and rat bone marrow by thinly spreading a drop of blood or bone marrow 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 were stained for 2 hours in Giemsa's stain (National Aniline, BSC Cert. No. NGe-16) and differentiated in 0.1 molar 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. Peripheral blood and bone marrow smears were fixed in the following solutions for 2, 8, 12, and 24 hours and then stained with Wright's or Giemsa's stain:

   10% formalin
   Absolute methanol
   Absolute ethanol
   Bouin's fluid
   Bouin's fluid followed by 1 hour in 70% ethanol
   5% metallic iodine in absolute methanol
   2% osmic acid

1041165

2. Fixed and unfixed peripheral blood and marrow smears were immersed in 1 and 2 molar concentrations of the following acids for 2 or 18 hours:

   hydrochloric acid
   hydrobromic acid
   phosphoric acid
   trichloroacetic acid
   nitric acid
   formic acid

3. After removal from the acid solution, the smears were either stained after a brief wash in distilled water or after being placed in Ringer's solution at pH 7.0 for 1 hour for Wright's stain or after being placed in 0.1 molar phosphate buffer at pH 7.2 for 1 hour for Giemsa's stain.

4. All slides were given a code number and the results analysed without knowledge of the precedures employed. Cytology and staining quality were judged separately and classified as Excellent, Good, Fair, or Poor. These classifications were defined before evaluation of the results as:

Cytology Excellent: Preservation of the cytological elements equal to the control slides. The granules were clear, distinct, rounded, and evenly distributed through the cell. The cells were filled without being swollen; the cells were not shrunken or irregular. Nuclear and cell membranes were distinct and intact. Nuclear chromatin was clear and either diffuse, or in blocks,

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or blocks with threads as was characteristic of the particular cell and fixative type. Red blood cells contained haemoglobin and were not shrunken or swollen.

Cytology Good: Slight deviations from the excellent results, such as indistinct or irregular granules, lack of clarity of the nuclear chromatin blocks or threads, irregular distribution of elements within the cells, slight vacuolization of the cytoplasm, or slight shrinkage of the cells.

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

Cytology Poor: Destruction of granules, nuclear chromatin or cellular membrane; gross shrinkage or swelling was present. End point was complete cellular disorganization.

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

Staining Good: Slight variations of color balance from the excellent classification. The red blood cells were palely stained, or unstained. In some sections, the red blood cells stained bluish. The background plasma of the nucleus and cytoplasm was overstained or

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irregularly stained. Granules showed variations in color intensity.

Staining Fair: Pale irregular staining with tendency to overstain with either the red or blue component. Differentiation was incomplete because of the overstaining of the cytoplasm and nucleoplasm.

Staining Poor: Cells stained with only one dye component and were without differentiation. Background heavily stained. Cells did not stain with the dye, or stained with odd combinations of unusual colors.

RESULTS:

The results of the combinations of fixative-acid-stain are summarized in Table I.

1. Effect of Fixation Without Acid Immersion: Good morphological preservation was obtained with all the fixatives tested. The optimal time for fixation was between 2 and 12 hours for blood smears; longer fixation made the cells more refractive to staining. Fixation in methanol, iodine in methanol, and, to a lesser extent, ethanol gave the best staining results with both Wright's and Giemsa's stain; smears fixed in 10% formalin were always overstained with the blue dye component; Bouin's fixation without 70% ethanol gave poor results with yellow red blood cells and loss of granules from the white cells. Fixation in Bouin's with 70% ethanol eliminated the yellow coloration of the cells from the picric acid and maintained the granules of the white cells, but the stain was still only fair. Staining

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following fixation with osmic acid was impossible because of the binding of the osmium to the cellular components. Morphological detail following osmic acid fixation was excellent.

2. Effect of Acid Immersion: Unfixed smears were completely removed from the slides by immersion in any concentration of any acid for 2 hours.

With fixed smears, no difference was found between 1 and 2 molar concentrations of hydrochloric, hydrobromic, and formic acids; the cytological results with these acids were good following fixation with formalin, Bouin's with ethanol, iodine in methanol, and 2% osmic acid. Distortion of cellular detail was found following immersion in 2 molar trichloroacetic or phosphoric acids; fair to good results were obtained in 1 molar concentrations. The results with nitric acid were always poor because of the destruction of cytological and staining qualities.

In general, good preservation of cytology was maintained following immersion in acids by fixation in formalin, osmic acid, Bouin's with 70% ethanol, and iodine in methanol; fair preservation was obtained following acid immersion after fixation in methanol; and poor preservation was obtained after fixation in Bouin's alone and ethanol.

The staining characteristics of the cells were as good as the preservation of the tissue by the fixative. If the cells

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were well preserved and the fixative gave good staining without acid immersion, the acid solution did not alter the results.

3. Description of the Cytological Results:

a. Red blood cells: Specific kinds of distortion of cytological detail and staining quality were associated with particular combinations of fixatives and acids. The results classified as follows:

Excellent: Red blood cells the same as 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 acid immersion regardless of fixation. This was corrected by buffering the smear for 1 hour in either Ringer's or 0.1 molar phosphate buffer. Following pH buffering, the red blood cells were most resistent to acid immersion following formalin or iodine in methanol fixation. Bouin's fixation with 70% ethanol gave fair preservation, but Bouin's alone gave poor results with colorless or shadowy cells. Ethanol and methanol fixation gave little protection against acid immersion and the cells appeared pale and swollen.

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Immersion of smears in hydrochloric, hydrobromic, and formic acids for 18 hours did not show any cytological alterations of the red blood cells after good fixation. The cells were usually pale and swollen after immersion in trichloroacetic and phosphoric acids regardless of the fixative; the alteration was greater in 2 molar than in 1 molar concentration. Nitric acid destroyed the red blood cells.

b. White blood cells: The white blood cells showed an order of resistance to alteration by acid immersion. The most resistant were the lymphocytes, then neutrophils, monocytes, and least resistant were the eosinophils. The ability of basophils to resist the effects of acid immersion could not be determined because of the limited number of cells.

In bone marrow, the blast cells were resistant to acid immersion but as the cells became progressively more mature, they were more easily altered by the acid solution.

The cytoplasmic elements also had a definite order of alteration during acid immersion. The most labile elements were the cytoplasmic granules; this was followed by swelling and alteration of the chromatin pattern of the nucleus; and finally fragementation of the cells and complete disorganization.

Fixation for 12 hours in formalin, iodine in methanol, and Bouin's with 70% ethanol protected the white cells from cytological damage during acid immersion, but not to the same degree with all the acids. Best results were obtained with hydrochloric,

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formic, and hydrobromic acids. The cytoplasmic granules were indistinct after immersion of smears in 2 molar trichloroacetic acid, but 1 molar concentrations did not cause any serious alteration.

Phosphoric acid produced clumping of the cytoplasmic granules of the eosinophiles and neutrophils which stained blue. Nitric acid produced cells which were pale staining, swollen, or completely disorganized.

c. Platelets: The platelets were well preserved in all acids except nitric acid. The best fixation method was 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 acid immersion on muscle and associated tissues.

Muscle tissues from male Wistar rats over 150 days of age were either perfused with or submerged in the 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 liquid. The skin was removed from the hind limbs of the rat and the muscles transected to make sections about 5 mm thick. The entire limb, with the attached muscle, was then submerged in fixing solution for at least 24 hours. Following fixation, muscle pieces 5 mm thick and about 1 centimeter in the other dimensions were cut from the limbs,

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washed in tap water, and placed in polyethylene containers with 100 ml of the acid solution. The polyethylene containers with the samples were agitated at 78 rpm on a roller agitator at 25° C. for either 24 or 48 hours. The muscle pieces were then washed in water, dehydrated in graded ethanol solutions, and embedded in 52° C. paraffin. Muscle sections 7 μ thick were cut and stained with haematoxylin and eosin or with a one-step trichrome (Morris[8]).

Five fixatives were used: 10% formalin, Bouin's fluid, Bouin's for 18 hours followed by 70% ethanol for 6 hours, iodine in methanol, and methanol. Tissues fixed in these fluids were placed in either 1 or 2 molar concentrations of the following acids: hydrochloric, hydrobromic, trichloroacetic, phosphoric, formic, and nitric acids. Fixed tissues not immersed in the acid solutions served as the controls.

RESULTS:

The slides were given code labels and the sections analysed without knowledge of what procedure had been applied. The slides were evaluated according to:

1) Preservation of morphological detail
2) Staining and color balance
3) Shrinkage
4) Tissue friability

All the nitric acid samples were discarded because the connective tissue was digested leaving the muscle fibers as a macerated brie. The results of the experiment are summarized in Table II.

Good morphological preservation and excellent staining

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were obtained in all the control slides except those fixed in methanol. Bouin's with 70% ethanol, 10% formalin, and iodine in methanol gave good protection to muscle and connective tissue during immersion for 48 hours in hydrochloric, formic, and trichloroacetic acids; poor preservation was obtained with these fixatives after immersion in phosphoric and hydrobromic acids.

Iodine in methanol gave excellent fixation results because the tissue appeared as if fresh. The muscle fibers were round, closely packed, and showed brilliant and numerous nuclei. The shrinkage was minimal with this fixative.

Formalin fixation appeared to predispose the tissue to being "cooked" during paraffin embedding. This was particularly true after immersion in hydrobromic, hydrochloric, and trichloroacetic acids. If the tissue was allowed more than the minimal time for paraffin infiltration, it would shred and shatter during sectioning.

Bouin's fixation without 70% ethanol produced poor nuclear staining and the connective tissue was swollen and partially digested following 48 hours immersion in any acid. The connective tissue change and lack of nuclear staining were eliminated by the additional fixation in 70% ethanol.

Staining results paralleled fixation. If the fixation was good, the staining was good; the tissue stained poorly in the center of the block where the fixation was imperfect. The

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staining of the nuclear chromatin with haematoxylin following immersion in the various acids was difficult and constituted the major alteration of morphological detail in the series.

EXPERIMENT 3: Effects of ion exchange resins and the electrolytic method on tissue preservation.

The caudal extremities of male Wistar rats over 150 days of age were fixed by perfusion or immersion with 10% formalin, Bouin's solution followed by 70% ethanol, or 5% iodine in methanol.

Four acids with four cation exchange resin combinations were used in the experiment. The acids were 1 molar concentrations of hydrochloric, formic, trichloroacetic, and phosphoric acids. Each acid was used in five combinations: as a control solution of acid alone, or with 25 grams of IRC 50+, or IR 112+, IR 120+, or Win 3000‡ resin. In all, 75 variations of fixative-acid solution-resin were used.

Before the extremities were placed in the demineralizing solutions, the muscle tissue was trimmed to about 1 centimeter thickness on all bone samples. Demineralization of the bone specimens was done under standard conditions in polyethylene containers with 100 ml of solution; the samples were agitated

+ Grateful acknowledgement is made to the Rhom and Haas Chemical Corporation who supplied the resins for this study.

‡ Win 3000 is distributed by Winthrop-Stearns, Inc. of New York City.

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at 78 rpm on a roller agitator at 25° C. until demineralized as determined by X-ray photography.

Following demineralization, the rat legs were washed in water, dehydrated in graded ethanol solutions, and cleared in 50% oil of Wintergreen-50% xylene. The tissues were embedded in 52° C. paraffin and 10μ sections cut and mounted. The tissues were stained with haematoxylin and eosin, one-step trichrome, Wright's and Giemsa's stains. The slides were buffered in Ringer's solution at pH 7.2 for 2 hours before staining.

Rat tissues were demineralized in an electrolytic bone machine at 25° C., with 15 watts electrolysis in 1 molar hydrochloric acid. This technique has been described previously by Morris and Benton[10].

RESULTS:

The histological results from this study are summarized in Table III. The chemical data have been previously reported (cf. Experiment 2, Morris and Benton[11]).

The histological results showed good tissue preservation and staining with formalin fixation and hydrochloric acid either with or without the addition of resins. Formic acid gave good results after formalin fixation; trichloroacetic gave fair results with formalin fixation, and phosphoric acid gave consistently poor staining results.

The slides fixed in Bouin's and 70% ethanol were generally

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poorer than those fixed in formaline. This was a difference in clarity of differentiation and staining rather than of morphological identification of the cellular elements.

Iodine in methanol gave very poor preservation of the blood cells of the bone marrow and the results are not recorded in Table III.

The tissue demineralized in the electrolytic machine was the same as that demineralized in the acid without electrolysis; no differences between the control and experimental samples could be demonstrated.

EXPERIMENT 4: Studies of methods of improving Wright's and Giemsa's stain of bone marrow after demineralization.

The femora from male Wistar rats over 150 days of age were removed and the ends of the bones split to expose the marrow cavity. The split bones were fixed in 10% formalin or Bouin's followed by 70% ethanol. The tissues were demineralized in 1 molar concentrations of trichloroacetic, hydrochloric, phosphoric, and formic acids and in 0.1 molar di-sodium di-hydrogen Versenate. The specimens were demineralized in 100 ml of solution while agitated at 78 rpm on a roller agitator at 25° C. Demineralization was determined by X-ray photography.

After demineralization, the specimens were washed in water, dehydrated in graded ethanol solutions, cleared in 50% oil of Wintergreen-50% xylene, and embedded in 52° C. paraffin. Tissue

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sections 7μ thick were cut and mounted. The tissue sections were immersed in one of the following solutions for 1 hour before staining with either Wright's or Giemsa's stain:

Phosphate buffer at pH 7.0
Ringer's solution at pH 7.0
Ringer's solution at pH 6.4
2% potassium dichromate and Ringer's solution at pH 9.0
2% potassium dichromate and Ringer's solution at pH 6.4
5% magnesium chloride
5% ammonium chloride
5% sodium chloride
2% sodium chloride
5% barium chloride

The sections were mounted in Clarite with coverslips after clearing in butyl alcohol and xylene.

RESULTS:

The results of the combinations of the variables in this experiment are shown in Table IV. Good cytology was obtained after fixation in either formalin or Bouin's with ethanol. Cellular staining comparable to the control blood smears was obtained with both Wright's and Giemsa's stain after demineralization in hydrochloric, formic, and trichloroacetic acids after the use of certain mordants.

Analysis of the results showed that out of 200 combinations of variables, excellent results in staining were obtained in 23 instances. These were after immersion in a mordant of either 2% potassium dichromate, or 5% sodium chloride, or 5% ammonium chloride. Poor results were obtained in 9 instances; these results were obtained after immersion in phosphate buffer, 2% sodium chloride, 5% magnesium chloride, or 5% barium chloride.

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Phosphate ion was associated with every poor result as either phosphate buffer or phosphoric acid. Tissues immersed in phosphate solutions were always overstained with the blue component of the dye mixture, with lack of staining with the red component. This result was associated with Wright's stain and occasionally with Giemsa's. Good staining was obtained with Giemsa's stain after phosphate buffer at pH 7.0 but no excellent results were obtained.

The analysis of the 23 excellent results showed that among the acids hydrochloric gave excellent staining 11 times, formic 6 times, and trichloroacetic 4 times. Phosphoric acid and Versenate were each classified as excellent in 1 instance. Excellent staining was obtained with Giemsa's method 14 times and with Wright's 9 times; excellent staining results were obtained after Bouin's ethanol fixation 14 times and after formalin fixation 9 times. Excellent staining followed the mordant in ammonium chloride 14 times, mordant in potassium dichromate 5 times, and mordant in 5% sodium chloride 4 times.

Thus from these data, the best combination of elements for excellent staining of bone marrow cells appears to be:

Fixation in Bouin's fluid followed by 70% ethanol
Demineralization in 1 to 2 molar hydrochloric acid
Mordant in 5% ammonium chloride
Staining in Giemsa's stain

EXPERIMENT 5: The application of the best combination of variables for rapid preparation of bone marrow slides

The purpose of this work has been to determine the most

1041179

reliable and rapid method of preparing excellent sections of decalcified bone for histological study. The following protocol is the record of a specific application of the results of this study:

Time in hours between operations	Manipulations of the tissue
0 hours	1. Surgical specimen of human rib removed and placed in fresh Bouin's solution.
2 hours	2. Rib specimen received from surgery and cut into cross sections with a Stryker bone saw; pieces were about 1 centimeter long and weighed about 2.5 grams. This type section maximized the surface area available to the demineralizing agent. Sections were returned to fresh Bouin's solution and placed on a roller agitator at 78 rpm.
4 hours	3. Specimens placed in 70% ethanol and agitated at 78 rpm.
2 hours	4. Specimens washed in tap water, placed in 100 ml of 1 molar hydrochloric acid and agitated on a roller machine at 78 rpm at 25° C.
3 hours	5. Demineralized specimens washed in tap water and agitated at 78 rpm during dehydration in graded ethanol solutions (50%, 70%, 95% and three changes of 100%). The specimens were cleared in 50% oil of Wintergreen-50% absolute ethanol, then in 50% oil of Wintergreen-50% xylene.
12 hours	6. Specimens embedded in 52° C. paraffin *in vacuo*, blocked, sectioned at 4 μ, mounted and hydrated.
2 hours	7. Sections immersed in 5% ammonium chloride for 30 minutes.

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1/2 hour

8. Wright's preparation made, dehydrated and cleared in butyl alcohol and xylene; coverslip with Clarite.

1/4 hour

9. Sections stained in Giemsa's stain, differentiated, and coverslip applied after clearing in xylene.

2-1/2 hours

RESULTS:

The staining and morphological results from this technique are demonstrated in Figures 1 and 2. Figure 1 shows the amount of shrinkage to be nil; Figure 2 shows the details of the marrow after Giemsa's stain.

The total time for preparation of the Wright's slide was 25-3/4 hours; the total time for the preparation of the Giemsa's slide was 28 hours.

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DISCUSSION

These experiments on staining of bone and associated soft tissues after demineralization have shown the critical element in any procedure to be the fixation of the tissues. If the tissues were well fixed, the cytology was not altered by the acid during demineralization. A fixative which gives good preservation during demineralization should have the following characteristics:

First, the fixative should be rapid in action because of the short time before blood cells show irreversible changes after removal of a bone piece or after the death of the animal. The density of the bone marrow and the limited amount of exposed surface necessitate that the fixative not only be rapid in action but also a rapid penetrant in order that it may reach the deeper tissue parts before they disintegrate.

Secondly, the fixative should not harden the tissue excessively because, with the hardening incident to the subsequent manipulations of acid immersion and paraffin embedding, frequently it is impossible to obtain tissue sections.

Thirdly, the fixative should be an agent which precipitates protein in an insoluble form. If the protein percipitate is not insoluble, excessive acid hydrolysis will occur during demineralization.

The advantages of various fixatives were observed by experimentation. Orth's fluid, Zenker's formalin, and Zenker's sublimate, despite their excellence as cytological preservatives,

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proved unsatisfactory as fixatives for preservation of bone marrow during demineralization. These fixatives were very slow penetrants and fixed only the outer millimeter or so of tissue, and, while this area has excellent preservation of cellular elements, it could not be used for study as it was torn up during the sawing of the bone sample. Additionally, dichromate fixatives made the tissue extremely hard, and after demineralization the sections tended to shatter and tear.

The use of metallic compounds such as iodine in methanol and osmic acid gave excellent fixation in thin smears or tissues which were reached during perfusion. The cells in the center of the bone block were usually disorganized after demineralization following iodine in methanol fixation, because the iodine did not reach the deeper areas of the bone marrow with sufficient rapidity to effect good fixation before the initial autolytic disorganization. The subsequent demineralization caused digestion of these unfixed tissues (cf. Experiment 3.) Osmic acid formed a complex with the tissue proteins which made subsequent staining impossible.

Alcohol solutions alone were unsatisfactory fixatives because they did not protect the tissue against acid hydrolysis. The nucleo-proteins were well fixed, and nuclear staining was generally good after demineralization. Fixation with alcohols was generally less effective in the center of the block than at the edge, although the alcohols were rapid penetrants. The less effective fixation at the center of the block may be due to the dilution effect of abosrbed water from the tissue during the penetration.

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Alcohol fixation did not harden the tissue excessively, but the protein precipitate was easily hydrolyzed by the acid solutions.

Formalin was found to be the best general fixative. It was rapid in penetration, did not harden the tissue excessively, and the protein compounds formed were resistant to acid hydrolysis. Low nitrogen values in the demineralizing solutions were characteristic of this fixative.

Bouin's fluid was a rapid penetrant and a soft fixative. It was an excellent protein precipitator, but many of the proteins remained water soluble so that with the change in pH incident to acid immersion they were hydrolyzed or lost. Bouin's alone did not fix the granules of the white cells or the nuclear chromatin, and these elements did not resist acid hydrolysis during demineralization. The use of ethanol as an additional fixative made these elements resistant, and the combined fixation gave excellent preservation during demineralization.

In the past, others have reported that Bouin's and formalin fixation gave poor staining of blood cells, and, because of this, other fixatives which were not as suitable for the purposes of demineralization but which gave better staining results were employed. But these fixatives did not protect the tissue from acid damage during demineralization. To correct this deficiency, methods were developed which used additives such as alcohol, olive oil, citrate salts, or phloroglucine to protect the tissue from acid alteration. These additives depressed the ionization of the acid and slowed both demineralization and tissue alteration.

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A similar result may be obtained by a low concentration of acid, but some tissue alteration also occurs with these solutions. In addition, low concentrations of acid have been found to cause excessive hydration of the tissue (cf. Morris and Benton[9]), and this can be as conducive to poor results as the higher acid concentrations. These additives have not proved as successful in preventing tissue alterations during demineralization as has good original fixation.

The addition of ion exchange resins to the demineralizing solutions did not improve either preservation of the tissue or the staining quality after demineralization. The electrolytic method of demineralization did not improve tissue preservation or staining quality when compared to the use of the same acid at the same temperature without electrolysis. If the temperature of the acid bath was increased beyond 40 C. during electrolysis, the tissue gave a poor staining reaction and appeared cooked. Electrolysis did not offer any advantages not found with hydrochloric acid alone.

The staining reaction with Wright's and Giemsa's stains after fixation in Bouin's-ethanol or formalin was improved by the use of mordant solutions. The clarity of staining of cytoplasmic granules and brilliance of red blood cells can be restored by immersion of the slide in 2% potassium dichromate for 1 hour. The use of 5% sodium chloride also restored good staining but the granular differentiation was not as good as with the dichromate solution. The best mordant was 5% ammonium chloride;

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it produced cell staining that could not be distinguished from the results obtained with air-dried smears of peripheral blood and bone marrow.

The results of this study have shown that many methods of demineralization are acceptable. If the tissue was well fixed with either Bouin's with ethanol or formalin, excellent results were obtained in hydrochloric, formic, and trichloroacetic acids. The best acid was determined to be a 1 to 2 molar concentration of hydrochloric because it gave a greater number of excellent results, a low nitrogen determination in the demineralizing soltuion, and was the most rapid demineralizer of all acids tested. Formic acid gave equally good results but was considerably slower and gave higher nitrogen values in the demineralizing solutions. Trichloroacetic acid proved to be a good demineralizer in a 1 molar concentration but a 2 molar concentration caused excessive cytological alteration.

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SUMMARY

1. It was found that cytological alteration during demineralization in hydrochloric, formic, and trichloroacetic acid can be prevented by good fixation with either 10% formalin or Bouin's followed by 70% ethanol.
2. Addition of ion exchange resins to the demineralizing solution did not alter the results in any way.
3. The acid of choice in demineralization is hydrochloric acid because it is the most rapid demineralizer, gave the greatest number of excellent results, and showed the least amount of nitrogen in the demineralizing solutions. Formic and trichloroacetic acid gave excellent results but were not considered as efficient as hydrochloric acid.
4. The use of 2% potassium dichromate or 5% ammonium chloride as a mordant for the tissues after demineralization restores the staining quality of formalin and Bouin's-ethanol fixed tissues. Wright's and Giemsa's stains after use of these mordants could not be differentiated from air-dried control slides.
5. Using the best possible combination of variables found in these studies, it is possible to demineralize and stain bone with Wright's stain in 26 hours or with Giemsa's stain in 28 hours.

1041187

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1041188

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1041189

TABLE I

Data showing the effect of fixation and immersion in various acids on the cytology and staining of blood and marrow smears as described in Experiment 1.

Fixative	Acid	Cytology	Wright's	Giemsa's	Fixative	Acid	Cytology	Wright's	Giemsa's
None	None	Ex	Ex	Ex	Bouin's with 70% Ethanol	None	G	F	F
	HCl	Smears removed during acid immersion				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
Formalin 10%	None	G	P	P	5% 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	Ex	Ex	Ex	2% osmic acid	None	Ex	P	P
	HCl	F	G	G		HCl	Ex	P	P
	HBr	F	F	F		HBr	Ex	P	P
	H_3PO_4	F	F	F		H_3PO_4	Ex	P	P
	CCl_3COOH	F	G	G		CCl_3COOH	Ex	P	P
	HNO_3	P	P	P		HNO_3	F	P	P
	HCOOH	F	G	G		HCOOH	Ex	P	P
Bouin's	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

1041190

TABLE II

Data showing the effect of fixation and acid immersion on cytology, and staining as described in Experiment 2. Ex - excellent, G- good, F - fair, P - poor.

Fixation	Acid	Cytology	Stain	
			H and E	Trichrome
Bouin's with 70% Ethanol	None	Ex	Ex	Ex
	HCl	G	G	F
	HBr	P	P	P
	H_3PO_4	F	F	P
	CCl_3COOH	F	F	G
	HCOOH	G	G	G
10% Formalin	None	Ex	Ex	Ex
	HCl	Ex	Ex	G
	HBr	F	F	P
	H_3PO_4	F	G	P
	CCl_3COOH	G	G	G
	HCOOH	G	Ex	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	Ex	Ex	Ex
	HCl	Ex	Ex	G
	HBr	G	G	G
	H_3PO_4	F	F	P
	CCl_3COOH	G	G	G
	HCOOH	G	Ex	G

1041191

TABLE III

Data showing the effect of fixation and demineralization in various acids and ion exchange resins on the cytology and staining of whole rats legs as described in Experiment 3.

Acid	Resins	Formalin					Bouin's				
		Cytology	H and E	Trichrome	Wright's	Giemsa's	Cytology	H and E	Trichrome	Wright's	Giemsa's
HCl	None	Ex	Ex	G	F	F	G	G	F	G	F
	IRC 50	Ex	Ex	F	P	F	G	G	G	P	F
	IR 112	Ex	Ex	G	F	F	G	G	P	F	F
	IR 120	Ex	Ex	F	F	F	G	G	F	F	F
	Win 3000	Ex	Ex	F	F	P	G	G	F	F	P
HCOOH	None	Ex	Ex	G	F	P	G	G	G	F	P
	IRC 50	Ex	Ex	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	Ex	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	Ex	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

1041192

TABLE IV

Data showing the effect of fixation, demineralization in various acids, and use of various mordants on cytology and staining of bone marrow as described in Experiment 4. W - Wright's stain, G - Giemsa's stain. Ex - Excellent, G - Good, F - Fair, P - Poor

Fixation	Acid	Cytology	Phosphate buffer pH 7.0		Ringer's pH 7.0		Ringer's pH 6.4		2% $K_2Cr_2O_7$ Ringer's pH 9.0		2% $K_2Cr_2O_7$ Ringer's pH 6.4		5% Magnesium chloride		5% Ammonium Chloride		5% Sodium chloride		2% Sodium chloride		5% Barium Chloride		Totals			
			W	G	W	G	W	G	W	G	W	G	W	G	W	G	W	G	W	G	W	G	Ex	G	F	P
Formalin	HCl	G	F	F	G	G	F	G	Ex	Ex	G	Ex	G	G	Ex	Ex	G	G	G	G	F	F	5	10	5	0
	HCOOH	G	P	G	F	G	G	F	G	G	G	G	G	G	Ex	Ex	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	Ex	Ex	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 + Ethanol	HCl	G	F	G	G	G	G	G	G	Ex	G	Ex	F	F	Ex	Ex	Ex	Ex	G	G	G	G	6	11	3	0
	HCOOH	G	F	F	G	G	G	G	G	G	G	G	F	F	Ex	Ex	Ex	Ex	G	G	G	G	4	12	4	0
	CCl_3COOH	F	F	G	G	G	F	G	G	G	G	G	F	F	Ex	Ex	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	Ex	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	Ex	G	G	F	F	G	G	1	11	8	0
	Ex								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			[illegible]	
	P		4										1	1						1	1	1				

1041193

Figure 1.

1041194

Figure 2., referred to on page 19, consists of two color plates of marrow sections which could not be reproduced in this report. They have been submitted for publication.

1041195