707918

STUDIES ON DEMINERALIZATION OF BONE*

III. The effect of ion exchange resins and Versenate in demineralization.

Russell E. Morris, Jr., and Robert S. Benton

With the technical assistance of

LaRose Smith and Jean Haskins

Department of Anatomy, The Medical College of South Carolina

Charleston, South Carolina

REPOSITORY Oak Ridge Operations Records Holding Area
COLLECTION Documents 1944-1994
BOX No. H-583-8 Bldg. 27144
FOLDER Grisely 1542 Univ. of S. Carolina

* This work was supported by Atomic Energy Commission

Contract AT-(40-1)-1542

1041145

Recently, attempts have been made to decrease the time necessary for demineralization of bone by adding various compounds to the demineralizing acid which bind and remove the dissolved bone minerals. The compounds used for this purpose have been ion exchange resins and chelating agents. Because both of these compounds employ the same principle in demineralization, they have been considered as a unit for this investigation of their usefulness.

Dotti, Paparo, and Clarke[3] introduced a method for demineralizing bone which used an ion exchange resin, Win 3000, mixed with formic acid. The method was reported to demineralize more rapidly than other methods and not to alter the morphology or staining reactions of the tissue elements. Lillie[4], in a review of this paper, questioned whether the results of the method were actually better than formic acid alone because there was no formic acid control for comparison. Case[2], in a study of the use of cation exchange resin in demineralization, found that addition of the ion exchange resin, Win 3000, to formic

1041146

acid did not appreciably shorten the demineralization time nor enhance tissue preservation and staining qualities.

Sreebny and Nikiforuk[7] and Birge and Imhoff[1] introduced a method of demineralization using another calcium acceptor. This method uses the chelating compound ethylene diamine tetra acetate or Versenate‡ in acid solution for demineralization. The method was admittedly slower than other methods, but it was still thought to be valuable because of the excellent preservation of the tissue elements.

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

---

‡ Versenate is the proprietary name given this compound by the Bersworth Chemical Company, Framingham, Massachusetts.

1041147

MATERIALS AND METHODS

1. Bone samples for the experiments were either (1) dense cortical bone from the shaft of an air-dried, adult, human femur or (2) whole caudal extremities of male Wistar rats over 150 days of age with the soft tissue intact. The samples of human bone were from the standard bone sample previously reported (Morris and Benton[5]).
2. All demineralizations were made in polyethylene containers and the samples were agitated at 78 rpm at 25° C. on a roller agitator.
3. 100 ml of acid solution of known molarity were used in each demineralization. In the resin experiments, 24 grams of resin in the saturated hydrogen form were added to 100 ml of acid solution of known molarity.
4. Four ion exchange resins were used for the experiments:
   1) IRC 50: a carboxylic acid type cation exchanger which operates in a pH range from 4.0 to 8.0.†
   2) IR 112: a sulfonic acid type cation exchanger which operates over the entire pH range.†
   3) IR 120: a sulfonic acid type cation exchanger which operates over the entire pH range.†

---

† Grateful acknowledgment is made to the Rohm and Haas Chemical Corporation who supplied the resins for this study.

1041148

4) Win 3000: an ammonium salt of a sulfonic acid type cation exchanger which operates in the acid range. ‡

5. The extent of demineralization was determined by X-ray photography.

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

---

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

1041149

EXPERIMENTS

EXPERIMENT 1: Comparison of demineralization using ion exchange resins and acid with the acid alone.

Five pieces of standard bone were shaped to approximately the same weight and surface area according to the method described in a previous paper (Morris and Benton[5]). The weights of the bone samples were from 235 to 244 mg and the surface areas from 165 to 169 sq. mm. One piece of bone was placed in each of the following solutions:

1 molar hydrochloric acid
1 molar hydrochloric acid + 25 grams of IRC 50 ion exchange resin
1 molar hydrochloric acid + 25 grams of IR 112 ion exchange resin
1 molar hydrochloric acid + 25 grams of IR 120 ion exchange resin
1 molar hydrochloric acid + 25 grams of Win 3000 ion exchange resin

The bone samples were agitated in these solutions until completely demineralized as determined by X-ray photography.

After the bone samples were completely demineralized they were digested in concentrated bydrochloric acid. Calcium, phosphorus, nitrogen, and molarity determinations were made on all the demineralizing solutions and on the digestion solutions.

RESULTS:

The bone samples were not demineralized in the same amount of time; the samples in the acid alone and the IRC 50 + acid were demineralized in 17 hours. The samples in IR 112, IR 120, and

1041150

Win 3000 + acid were demineralized in 19 hours.

The weight loss for all the bones was about the same, but the least weight loss was found in the samples demineralized in the acid alone, IR 112, and Win 3000 + acid. These samples lost about 105 mg of their weight. The samples demineralized in IR 120 and IRC 50 had a weight loss of about 111 mg or 6 mg more than the other samples. These data are shown in Table I.

There was no significant change in the acid molarity of any solution during demineralization; nitrogen determinations on the demineralizing solutions showed less than 0.1 mg nitrogen for all samples. Unbound mineral is recorded in Table I.

The nitrogen determinations on the digestion mixture of the demineralized bone pieces showed no difference in the amount of protein hydrolysis between the acid and resin solutions. All determinations were within 2 parts per 1000 of each other. No calcium or phosphorus could be detected in the digestion solutions. These data are recorded in Table I.

EXPERIMENT 2: <u>Comparison of demineralization with ion exchange resins and acid with acid alone using bone and associated soft tissues</u>.

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

Four acids with four resin combinations were used in the experiments. The acids were 1 molar concentrations of

1041151

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, or IR 120, or with 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 a thickness of about 1 centimeter on all sides of the bone samples. Complete demineralization was determined by X-ray photography in all samples except those fixed in iodine solution. The presences of the iodine in the tissues made the specimens opaque and masked the removal of the calcium from the bone. After demineralization, the extremities were sectioned for microscopic examination. These results will be reported in a subsequent paper.

Calcium, phosphorus, nitrogen, and molarity determinations were made on the demineralizing solutions before and after demineralization.

RESULTS:

The order of demineralization of the various samples may have been due to the difference in the size of the extremities and the amount of muscle mass although the specimens were selected at random. In general, the control, the acid alone, finished first most frequently; the resin solutions IRC 50 and IR 112 usually finished early, Win 3000 finished late as

often as it finished early and IR 120 was the solution most frequently last to demineralize the sample.

Changes in the molarity of the acid solution were slight being less than 0.2 molar in the control and IRC 50 resin solutions, and less than 0.1 molar in the other resin solutions.

Nitrogen determinations showed that most nitrogen was removed from the tissue during demineralization by trichloroacetic acid; phosphoric acid was next, and then formic acid. The least amount of nitrogen removed was by hydrochloric acid. The amount of nitrogen removed by an acid was the same with or without the addition of resins. The average nitrogen removed by the various acids is recorded in Table II.

EXPERIMENT 3: Comparison of alkaline and acid Versenate † as demineralizing agents with 1 molar hydrochloric acid.

Samples of standard bone were shaped to approximately the same weight and surface area as previously described (Morris and Benton[5]).

Data:

Sample	Weight (mg)	Surface Area (sq. mm.)
1	85	106
2	88	105
3	85	105

† Grateful acknowledgement is made to the Bersworth Chemical Company of Framingham, Massachusetts who supplied the Versenate for these studies.

1041153

Sample 1 was placed in a 1 molar concentration of hydrochloric acid; sample 2 was placed in a 0.1 molar (saturated) solution of di-sodium di-hydrogen Versenate at pH 4.8; sample 3 was placed in a 0.1 molar (saturated) solution of tetra-sodium Versenate at pH 8.8. The tetra-hydrogen form of Versenate was not used because it is almost insoluble at the higher pH values.

Complete demineralization was determined with X-ray photography. Nitrogen determinations were made on all solutions.

RESULTS:

Sample 1 in 1.0 molar hydrochloric acid was demineralized in 4 hours; sample 2 in di-sodium di-hydrogen Versenate was demineralized in 12 days; sample 3 in tetra-sodium Versenate was not completely demineralized in 25 days when the experiment was terminated. The amount of nitrogen in all solutions was nil.

1041154

DISCUSSION

The purpose in using an ion exchange resin for demineralization is two-fold (Dotti et al[3]): to decrease the time necessary for demineralization, and to decrease the amount of soft tissue destruction by the hydrolytic action of the acid. Ion exchange resins will accept and bind cations; by removing the cation Ca^{++}, it was believed that the diffusion gradient of the bone salts out of the bone would be increased, and this would decrease the time necessary for demineralization. The use of ion exchange resin Win 3000 was reported to prevent morphological alteration and to maintain the typical staining reactions of the cellular elements. The mechanism of protection was not defined by the innovators of the method.

All cation exchange resins do not accept the same amount of calcium ion per gram of weight. Titrations of resins with calcium ion showed that IR 120 and Win 3000 were the most efficient calcium acceptors and bound about 28 mg Ca^{++} per gram of resin; IR 112 was intermediate in capacity and bound about 18 mg Ca^{++} per gram of resin; IRC 50 had the least capacity and bound less than 1 mg Ca^{++} per gram in acid solution.

In Experiment 1, standard bone samples were demineralized in mixtures of hydrochloric acid and resins. The results showed IRC 50 to be the most rapid resin demineralizing system; this resin accepts the least amount of calcium ion of the resins

1041155

studied. In Experiment 2, where resins and acids were used for demineralizing bone with associated soft tissues, IRC 50 and IR 112 were the most rapid resin demineralizing systems; again, these resins are low calcium ion acceptors. The resins with the highest calcium ion binding capacity were slower demineralizing systems. In both Experiments 1 and 2, the resin-acid mixtures did not demineralize as rapidly as did the acid solution alone.

Protection of the tissues by resins could not be demonstrated. The amount of nitrogen in the solution after demineralization indicates the amount of protein hydrolysed by the acid solution. If the resin solution protects the soft tissues from the acid hydrolysis, the amount of nitrogen in the resin solutions should be less than that found in the control acid solutions. In Experiment 1, the amount of nitrogen in the solutions after complete demineralization was the same for both the acid control and the acid-resin solutions. The amount of nitrogen in the digested demineralized bone samples was the same for the control acid solution and the acid-resin solutions. In Experiment 2, where the caudal extremities of Wistar rats were demineralized in combinations of acid-resin-ficative, the muscle bulk offered almost unlimited protein for acid hydrolysis; the amount of nitrogen removed from the samples was the same for all the fixative-resin combinations with a given acid. A difference in the amount of nitrogen released from the tissue was found

1041156

but this was associated with the type of acid used. Trichloroacetic acid removed the most nitrogen from the tissues and hydrochloric acid removed the least amount of nitrogen from the tissues (cf. Table II).

Thus, it has been found in these experiments that resins do not decrease the time necessary for demineralization. Resins do not offer protection to the soft tissue parts as demonstrated by the nitrogen determinations because the amount of nitrogen removed by an acid was the same with or without the addition of resins.

The use of the chelating agent, Versenate, was found to be ineffective because of the very slow rate of demineralization. Acid Versenate does not offer any advantages which are not found in hydrochloric acid, but does offer the disadvantage that it takes 70 times as long to demineralize.

One of the advantages of Versenate according to the report of Sreebny and Nikiforuk[7] was that it could be used as a neutral or alkaline demineralizing agent. Experiment 3 showed that demineralization, for all practical purposes, does not take place in alkaline Versenate solution. Thus, Versenate to be effective must be in the acid form and therefore presents the same difficulties as any weak acid solution.

In previous papers (Morris and Benton[5,6]) it was demonstrated that the speed of demineralization is determined by the dissociation constant of the acid used to break down the bone mineral. If a

1041157

resin is added to the demineralizing acid, it does not alter the amount of hydrogen ion present by accepting the calcium salts. Further, the resin does not protect the tissue from the hydrolytic action of the acid by accepting the free cations. The resin may protect the tissue to a limited extent by removing the hydrogen ion from solution and reducing the acid concentration, but this was improbable in the experiments because the resins were in the saturated acid form initially, and in this state will accept almost no additional hydrogen ion. This was demonstrated by titrations of the acid molarity before and after addition of the resins to the solution; no difference in molarity could be determined.

Hydrogen ion is the responsible agent for demineralization of bone and for the alterations in morphology and staining qualities of the tissues. The resins or chelating agents are additives which remove calcium and other cations from the solution but are ineffective in demineralization.

1041158

SUMMARY

1. Four cation exchange resins in acid solutions were studied to determine their effect on demineralization. The resins did not decrease the time necessary for demineralization below the time necessary for demineralization with the acid solution alone.
2. Ion exchange resins do not offer any protection to the soft tissues during demineralization. The amount of nitrogen removed from the samples was the same with or without addition of the resins to the acid solution.
3. Versenate is a weak acid demineralizer similar to acetic acid in action. While it does not produce tissue destruction, it greatly increases the time necessary for demineralization. It does not offer any advantages which are not present in hydrochloric acid.
4. Versenate demineralizes bone in alkaline solution extremely slowly.

1041159

BIBLIOGRAPHY

1. Birge, E. A., and Imhoff, C. E.: Versenate as a decalcifying agent for bone, Am. J. Clin. Path., 22: 192-193, 1952.

2. Case, N. M.: The use of a cation exchange resin in decalcification. Stain Techn., 28: 155-158, 1953.

3. Dotti, L. B., Paparo, G. P., and Clarke, B. E.: The use of ion exchange resin in decalcification of bone. Am. J. Clin. Path., 21: 475-479, 1951.

4. Lillie, R. D.: Review. Laboratory hints from the literature. Stain Techn., 26: 276, 1951.

5. Morris, R. E., and Benton, R. S.: Studies on demineralization of bone. I. The basic factors of demineralization.

6. Morris, R. E., and Benton, R. S.: Studies on demineralization of bone. II. The effect of electrolytic techniques in demineralization.

7. Sreebny, L. M., and Nikiforuk, G.: Demineralization of hard tissues by organic chelating agents. Science, 113: 560, 1951.

1041160

TABLE I

Data showing weight loss, percent weight loss, milligrams of calcium, phosphorus and nitrogen in the solution after complete demineralization of standard bone samples of similar weights and surface areas in solutions of hydrochloric acid and ion exchange resins as described in Experiment I.

Time of Demineralization (Hours)	Demineralizing Solution	Bone Weight (mg)	Bone Weight Loss (mg)	% Weight Loss	Mineral in Solution (mg) Ca	P	Nitrogen in Solution (mg)	Nitrogen in demineralized bone matrix after complete digestion (mg)
17	1 M HCl	240	104	43%	66	23	Less than 0.1	9.1
	1 M HCl + 25 g. IRC 50	244	112	46%	59	21	Less than 0.1	9.5
19	1 M HCl + 25 g. IR 112	235	103	44%	27	21	Less than 0.1	9.1
	1 M HCl + 25 g. IR 120	241	111	46%	12	22	Less than 0.1	9.2
	1 M HCl + 25 g. Win 3000	238	105	44%	11	22	Less than 0.1	9.5

1041161