Demineralization in Organic Solvents by Alkylammonium Salts of Ethylenediaminetetra-acetic Acid

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1. Insoluble salts of alkaline earth metals (e.g. calcium phosphate) dissolve in non-aqueous solvents containing EDTA salts of organic cations (e.g. tetraethylammonium).
2. Bone is efficiently demineralized in ethanolic trimethylammonium EDTA. Retention in the matrix of water-soluble materials (e.g. proteoglycan) is much better than in standard aqueous EDTA demineralization procedures.

EDTA is a tetravalent anion. The chelate complex with, e.g., calcium still retains two negative charges. Both chelator and complex are therefore present as salts. The salts normally used (e.g. Na^+) are very soluble in water but insoluble in organic solvents. By replacing, e.g., Na^+ with an organic cation, it is possible to produce both a chelator and a complex that are soluble in organic solvents. We have found that tetraethyl-, tetramethyl-, triethyl- and trimethylammonium salts of EDTA are very soluble in ethanol etc., and that efficient chelation of Ca^{2+} takes place in the resulting solutions. Solid samples of water-insoluble salts of calcium (CaCO_3 , CaSO_4 , CaHPO_4) and barium (BaCO_3 , BaSO_4) are easily soluble in these solutions.

Demineralization of biological materials has generally been conducted in aqueous media, resulting in considerable loss of water-soluble material (Herring *et al.*, 1974), gross disruption of macromolecular organization and chemical breakdown of many components of biological tissues. By working in organic solvents, loss of water-soluble materials should be much less, and preservation of structures should be better. Many organic media are relatively inert compared with aqueous media, and chemical breakdown should therefore be minimized. We present results on the development of a practical system for the demineralization of bone, which may also have wide application to other biological systems.

Materials and Methods

Materials

CaCO_3 , $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$, BaSO_4 and BaCO_3 were Analar from BDH Chemicals, Poole, Dorset, U.K. Freshly precipitated CaHPO_4 was prepared by mixing equal volumes of 0.1M solutions of CaCl_2 and

Na_2HPO_4 . The precipitate was washed with water, then three times with test solvent.

Aq. tetraethylammonium hydroxide (25%, w/v), aq. trimethylamine (25–30%, w/v), triethylamine (BDH Chemicals), aq. tetramethylammonium hydroxide (25%, w/v) and ethylenediaminetetraacetic acid (Fisons Scientific Apparatus, Loughborough, Leics., U.K.) were laboratory grade. A.R. disodium EDTA was from Fisons Scientific Apparatus.

Chondroitin sulphate D (Miles Laboratories, Maidenhead, Berks., U.K.) and sodium alginate (BDH Chemicals) were commercial samples. Sodium hyaluronate was prepared by the method of Scott (1960) from mesothelioma fluid.

Armeen M2C was from Armour Hess Chemicals, Leeds, U.K.

Alkylammonium EDTA solutions

A solution (0.2M) of the trimethylammonium EDTA was prepared by stirring ethylenediaminetetraacetic acid (0.2mol, 58.5g) with ethanol (780ml) and adding triethylamine solution dropwise until all the acid had dissolved and 1 drop of the solution yielded a faint colour (approx. pH 9.0) with 1 ml of aq. thymol phthalein solution. The volume was diluted to 1 litre with water. The amount of base added at this end point corresponds to slightly less than that calculated to produce 100% of tetrakis(triethylammonium) EDTA.

Tetramethylammonium, tetraethylammonium and trimethylammonium salts were prepared in the same way.

Solutions of other concentrations can be made and formalin can be included, and a final water content of 20% can still be maintained.

Solutions of triethylammonium EDTA (0.1M) in dimethylformamide/water (7:3, v/v) and dimethyl

sulphoxide/water (3:1, v/v) were prepared in a similar manner.

Bone

Fresh rat tibias were washed with ethanol. Fresh bovine shin bone was washed with ethanol and then ground in a Hughes press under liquid N_2 . The powdered bovine bone was sieved into three sizes: (a) material passing a screen mesh with 40 holes per square inch; (b) material retained by this sieve but passing a screen sieve with holes of 1 mm diameter; (c) material retained by the 1 mm screen but passing a screen sieve with holes of 5 mm diameter.

Methods

The EDTA concentration of the reagent solutions was determined by titration against 0.1 M- $Ca(NO_3)_2$ in pH 10.5 (approx.) aq. NH_3 (28%, v/v; 0.880 sp.gr.)/ NH_4Cl (3.5%, w/v) buffer, with Eriochrome Black T as indicator.

Calcium was determined by using a Shandon Southern A3300 atomic absorption spectrophotometer with Analar $Ca(NO_3)_2$ standards (0.5–3.0 p.p.m.).

Hexosamine was determined by the method of Kraan & Muir (1957) on hydrolysates of bone or matrix. The samples (0.5–2.5 g) were dissolved at 102°C in 20 ml of HCl/water (1:1, v/v) in 1 h, and were then diluted to 25 ml or 50 ml with 50% (v/v) HCl. Samples (5 ml) of these solutions were further hydrolysed in sealed tubes at 102°C for 16 h, and acid was extracted into 50% (v/v) Armeen M2C in chloroform (Scott & Newton, 1975).

Hydroxyproline was determined by an automated modification (J. Weiss, unpublished work) of the method of Woessner (1961) on the neutralized hydrolysates.

Uronic acid was determined by the carbazole method (Bitter & Muir, 1962) and chondroitin sulphate by cetylpyridinium chloride titration (Scott, 1960) on papain digests of demineralized bone (Scott, 1960). Papain digests were hydrolysed with HCl for hexosamine, hydroxyproline and calcium determination. Corrections were applied, if necessary, for contributions from the papain.

Dissolution of water-insoluble calcium and barium salts

Samples (0.05 mmol) of the solids were shaken with solutions of the alkylammonium EDTA salts at room temperature (20°C) in tubes (10 ml capacity) in a rotary mixer at 6 rev./min. The tubes were inspected at regular intervals for 2–3 h.

Demineralization of bone samples

Rat tibias (100–300 mg) were mixed at room temperature or stirred at 40°C with ethanol/water

solutions of the alkylammonium EDTA salts for periods up to 30 days. Samples of the supernatant solution were removed periodically and residual EDTA-chelating capacity was determined.

Weighed samples (1.0–2.5 g) of bovine bone were treated with trimethylammonium EDTA in ethanol/water solutions (containing 5% formalin) for periods up to 32 days and at temperatures up to reflux (78°C). Samples of bovine bone were similarly demineralized in aq. 0.1 M-sodium EDTA adjusted to pH 6.9–7.1 with NaOH. Uncomplexed EDTA was determined as above.

Results

Dissolution of water-insoluble calcium and barium salts

Samples of the calcium salts and $BaCO_3$ were treated with an equivalent or an excess of 0.1 M-tetraethylammonium EDTA in ethanol/water (4:1, v/v). $CaHPO_4$ and $CaSO_4$ readily dissolved (in about 20 min and 80 min respectively), and $CaCO_3$ and $BaCO_3$ were almost completely soluble after 2 h. Dissolution was faster in an excess of reagent.

$CaHPO_4$ and $CaSO_4$, mixed with 0.1 M solutions of tetraethylammonium, tetramethylammonium, triethylammonium and trimethylammonium EDTA in ethanol/water (4:1, v/v) took approximately the same time to dissolve. Increased concentrations (0.67 M or 1.0 M) of triethylammonium EDTA in ethanol/water (4:1, v/v) dissolved $CaHPO_4$ more rapidly. However, the 0.67 M solution dissolved $CaSO_4$ faster than the more viscous 1.0 M solution.

Inclusion of formalin in the EDTA solutions did not affect the rate of solution.

In 3 h, $CaHPO_4$ and $CaSO_4$ dissolved completely and $CaCO_3$ dissolved partially in 0.1 M solutions of triethylammonium EDTA in ethanol/water (4:1, v/v), dimethylformamide/water (3:1, v/v) and dimethylsulphoxide/water (7:3, v/v). The ethanolic solution was the most effective. $BaCO_3$ and $BaSO_4$ dissolved almost completely in the ethanolic solution, partially in the dimethylformamide solution and only slightly in the dimethyl sulphoxide solution.

Solubility of polysaccharides

Samples (5 mg) of chondroitin sulphate D (sodium salt), sodium alginate and sodium hyaluronate, left for 48 h at room temperature in 2 ml of 0.1 M-triethylammonium or trimethylammonium EDTA in ethanol/water (4:1, v/v) with and without formalin, swelled only slightly and least in the trimethylammonium EDTA solution. At 40°C, swelling was slightly greater. Swelling was lessened when the water content was decreased to 10% (v/v). The swelling of chondroitin sulphate D was still low in this solvent

Table 1. *Effect of time, temperature and bone-particle size on the extent of demineralization and composition of residual bovine bone matrix after demineralization with 100 ml of 0.2M-trimethylammonium EDTA in ethanol/water (4:1, v/v) containing 5% formalin*

% demineralization is measured as the decrease in complexing capacity of EDTA solution. Theoretical 100% = 6.54 mmol/g.

	Time (days)	Temperature (°C)	Bone particle size	Demineralization (%)	Residual content in matrix (assuming 100% recovery of bone sample)			
					Calcium (mg/g of original bone)	Hexosamine (μg/g of original bone)	Hydroxyproline (mg/g of original bone)	Hexosamine/hydroxyproline (μg/mg)
Bone (1 g)	1	20	< 40 mesh	17	—	—	—	—
	1	20	40 mesh to 1 mm	9	—	—	—	—
	1	20	1–5 mm	8	—	—	—	—
	1	50	< 40 mesh	21	—	—	—	—
	1	50	40 mesh to 1 mm	14	—	—	—	—
	1	50	1–5 mm	12	—	—	—	—
	1	Reflux	< 40 mesh	70	—	—	—	—
	1	Reflux	40 mesh to 1 mm	79	—	—	—	—
	1	Reflux	1–5 mm	47	—	—	—	—
	16	20	< 40 mesh	—	50	960	20.8	46
	16	20	40 mesh to 1 mm	—	60	1100	24.8	44
	16	20	1–5 mm	—	100	1160	27.2	43
Bone (2 g)	1	60	< 40 mesh	54	—	—	—	—
	1	60	40 mesh to 1 mm	49	—	—	—	—
	1	60	1–5 mm	31	—	—	—	—
	13	60	< 40 mesh	—	18	1020	18.5	55
	13	60	40 mesh to 1 mm	—	10	1320	24.0	55
	13	60	1–5 mm	—	50	1180	24.0	49
Untreated bone	—	—	—	—	200	1420	26.0	55

Table 2. *Composition of residual bovine bone matrix after demineralization of 2.5 g of 1 mm to 40 mesh bone with 250 ml of 0.2M-trimethylammonium EDTA in ethanol/water (4:1, v/v) or 5% aq. disodium EDTA for 25 days at 20°C*

Demineralization solvent	Chondroitin sulphate (recovered after papain digestion)		Calcium (mg/g of original bone)	Hexosamine (μg/g of original bone)	Hydroxyproline (mg/g of original bone)	Hexosamine/hydroxyproline (μg/mg)
	Cetylpyridinium chloride titration (mg/g)	Uronic acid (μg/g)				
Ethanol (80%)	1.05	270	1.3	1300	30.5	43
Ethanol (80%)	0.90	220	1.3	1320	32.0	41
Water	No sharp end point	25	0.7	880	30.0	29
Untreated bone	—	—	200	1300	31.0	42

at reflux temperature. The extent of swelling was not influenced by the presence of formalin.

Demineralization of bone

Preliminary experiments showed that whole rat tibias, after treatment with tetraethylammonium, triethylammonium and trimethylammonium EDTA in ethanol/water (4:1, v/v) at room temperature over a period of 30 days, lost 95–100% of calcium.

The results of varying the temperature, particle size and time on the demineralization of bovine bone with 100 ml of 0.2M-trimethylammonium EDTA (with 5% formalin) in ethanol/water (4:1, v/v) are shown in Table 1, which includes analysis of the residues (as well as original bone) for hexosamine, hydroxyproline and calcium. A comparison of aqueous and 80% ethanol EDTA is shown in Table 2. The kinetics of demineralization at room

Fig. 1. Kinetics of demineralization of bovine bone at room temperature in 0.2M-triethylammonium EDTA (with 5% formalin) in ethanol/water (4:1, v/v)

The bone particle sizes are 5-1 mm (●), 1 mm to 40 mesh (▲) and < 40 mesh (■).

Fig. 2. Kinetics of demineralization of bovine bone at 40°C with 0.1M-EDTA solutions containing 5% formalin. Trimethylammonium EDTA in ethanol/water (9:1, v/v) (●) and aq. sodium EDTA at pH 6.9-7.1 (▲) (with 1-5 mm particle size bone), renewed at the points indicated by an arrow.

temperature of bone particles of various sizes is illustrated in Fig. 1, and Fig. 2 compares the kinetics of demineralization of 2g samples of bovine bone (1-5 mm) in ethanolic and aq. 0.1M-EDTA solutions (containing 5% formalin) at 40°C

Demineralization was more than 80% complete in most cases within a period of 30 days and almost complete at reflux in 1 day in some cases.

Comparison of the contents of hexosamine and hydroxyproline in demineralized matrix with that of untreated bone (Tables 1 and 2) shows little or no loss into ethanolic EDTA, whereas considerable amounts of hexosamine are lost into aq. EDTA. Calculations are based on assumed 100% recovery of the sample. The very fine particles in samples (a) were not easily recovered, and hence the ratio hexosamine/hydroxyproline is more informative.

About 10 times as much chondroitin sulphate was recovered from the residues of ethanolic demineralization (Table 2) compared with that from the aqueous demineralization procedure.

The chondroitin sulphate migrated on electrophoresis in barium acetate buffer (Newton *et al.*, 1974) at the same speed as standard chondroitin 4-sulphate.

Discussion

The prospect of producing chelator and complex as salts that are soluble in a wide range of solvents opens a new approach to the dissolution of inorganic solids and to the removal of mineral from biological specimens. In this investigation we have found that a range of organic bases from trimethylamine to tetraethylammonium hydroxide form EDTA salts that are soluble in dimethylformamide, dimethyl sulphoxide and ethanol. We have produced a chloroform solution of the *NN*-didodecyl-*N*-methylammonium salt of EDTA in which solid CaHPO_4 is soluble.

Since our immediate aim is to demineralize bone, we have examined specific properties of these solvents and EDTA salts that are relevant to the production of a matrix containing the macromolecular components in a form suitable for ultrastructural and biochemical investigation. It is desirable to remove the mineral and to allow non-chelated cations (e.g. Na^+ , K^+) to escape into the supernatant, but not, in the process, to remove mucopolysaccharides and other materials. In this context ethanol with a small percentage of water is very suitable. Anionic polysaccharides did not dissolve in this solvent in the presence of trimethylammonium or triethylammonium EDTA at temperatures up to reflux. Solution of inorganic material was stoichiometric, at a rate comparable with that of completely aqueous solutions. The speed can be varied by changing the temperature or concentration of EDTA (which is soluble at a concentration less than 1M in the case of the trimethylammonium salt).

Bone can be demineralized effectively at room temperature in 80% aq. ethanol containing 0.2M trimethylammonium EDTA. By increasing the

temperature, a very rapid demineralization can be achieved, and even larger pieces of bone were more than 50% demineralized in 1 day at reflux. As might be expected, the speed of the demineralization is affected by the extent of the surface area presented to the reagent. Nevertheless, even after the initial rapid attack on the outer layers, the reagent is still able to extract mineral from the interior of samples at a practical rate. Elevated temperatures may lead to denaturation of collagen.

Analysis of the residual matrix shows that no appreciable amount of collagen is lost even at 60°C over a period of many days, nor is the content of hexosamine seriously decreased. The ratio of hexosamine/hydroxyproline in the residue is practically the same as that of untreated bone.

After using aqueous EDTA solutions, it is difficult or impossible to work on the localization and distribution of water-soluble materials such as the glycosaminoglycans in bone matrix. Herring *et al.* (1974) showed that aq. EDTA at pH 7.5 extracts over 60% of the non-collagenous protein of bone. The recovery of chondroitin sulphate from bovine bone completely demineralized in 80% ethanolic reagent is about 10 times that from bone demineralized in standard aq. EDTA. Since the polysaccharides are completely insoluble in our reagent, we expect that the histological and ultrastructural examination of bone will now become possible with the intention of showing relationships between polysaccharides and collagen or cells etc. Preliminary results obtained on rat tibia, decalcified according to these principles, show good preservation of structure.

Earlier workers (e.g. Jenkins, 1921) have empiri-

cally concocted decalcification solutions containing organic solvents, invariably including strong acids that adversely affect the tissue, and the macromolecules therein.

Our proposed reagent, trimethylammonium EDTA in 80% ethanol, is inexpensive and convenient and should facilitate demineralization of a very wide range of biological structures at the macroscopic and submicroscopic levels. It has previously been particularly difficult to examine submicroscopic structures for the presence of water-soluble materials, after conventional EDTA demineralization. It will be clear that, by using solvents with properties different from those of the 80% ethanol solution, other materials could be retained, or alternatively extracted, in a specific way, during the demineralization process.

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