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IMPROVED TISSUE CORROSION OF VASCULAR CASTS: A QUANTITATIVE FILTRATION METHOD USED TO COMPARE TISSUE CORROSION IN VARIOUS CONCENTRATIONS OF SODIUM AND POTASSIUM HYDROXIDE

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Abstract

In this study, we compare weights of filter retained material (11 μm particle retention) after solubilization and filtration of unfixed, un-perfused tissue (fat, liver and trachea) in sodium and potassium hydroxide (1%, 5%, 10% and 20% weight/volume, w/v) at 8, 24 and 48 hour time points at 45°C. Three detergents [1% Triton-X-100 (volume/volume, v/v), 1% 7X (v/v), 1% Terg-A-Zyme (w/v)] used in combination with hydroxide were evaluated for use in solubilizing fat. Additionally, vascular casts from mouse kidneys were corroded to test the practical effectiveness of corroding solutions on resin infused tissue.

Five percent KOH for eight hours proved to be the most effective concentration and time required to corrode fatty tissue. Liver tissue was corroded most rapidly in 1% to 5% NaOH or in 1% to 20% KOH. Corrosion of trachea tissue showed that 5, 10 and 20% hydroxide (NaOH or KOH) are equally effective after 8 hours of corrosion. Use of detergents improved solubilization of fat when combined with 2%, 3% or 5% NaOH. However, fatty tissue was solubilized more rapidly without the use of detergents in 1% NaOH. Scanning electron microscopy of vascular casts shows that corrosion in 1% NaOH appears equally as effective as corrosion in 15% KOH.

Key Words: Corrosion casting, tissue maceration, scanning electron microscopy.

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Introduction

Corrosion casting is a useful technique for examining both macro- and microvasculature. Our group has used corrosion casting to examine vasculature in a number of tissues, including murine lymph nodes (Steeber *et al.*, 1987), mammary ductal organization (Shenkman *et al.*, 1985) tumor vasculature and vascular response to implanted materials (unpublished). The process involves flushing the vasculature with a lavage medium which contains an anti-coagulant with or without a vasodilator. The vasculature is then filled with a low viscosity partially polymerized methacrylate resin which quickly polymerizes to form a cast surrounded by tissue. The desired area of interest is carefully removed and the biological material surrounding the cast is corroded away to reveal polymer casts of veins and arteries with characteristic imprints of cell surfaces including readily identifiable nuclear imprints and endothelial cell border lines. This technique has been helpful in such diverse studies as hormone transport in the pineal gland (Hodde, 1979), nasal circulation and temperature regulation (Hodde, 1986), hypertension (Hodde *et al.*, 1984), immunology (Steeber and Albrecht, 1992), neovascularization (Grunt *et al.*, 1986), tumor vasculature (Walmsley *et al.*, 1987), as well as in a variety of other applications (e.g., see, Konerding, 1991; Motta *et al.*, 1992).

Although scanning electron microscopy has been used to image vascular casts for over 20 years, there are few standard protocols with respect to the maceration of cast specimens, (Lametschwandtner *et al.*, 1990). Differences in procedure vary depending upon differences in composition, vascular density and treatment prior to maceration or corrosion (Lametschwandtner *et al.*, 1990). Schraufnagel (1989) showed the use of proteolytic enzymes, detergents, or warm tap water before the alkali treatments did not significantly improve corrosion. Corrosion or digestion of tissue from vascular casts is generally performed using 7.5% to 40% hydroxide. Acids, enzymes, proteolytic detergents, and sonication have also been used either alone or in combination with other agents (Lametschwandtner *et al.*, 1990, Hodde *et al.*, 1990; Schraufnagel, 1989).

We have previously employed 20% NaOH

(Steeber *et al.*, 1987) and 7.5% NaOH with 5% Triton (Steeber and Albrecht, 1992) to corrode tissue surrounding casts of lymph node vasculature. However, additional corrosion and rinsing in distilled water was occasionally required to remove the firm white material that formed in place of the fatty tissue. We believed that the saponification of fats and aggregation of associated micellar structures were responsible for the development of the corrosion resistant material. Lower concentrations of hydroxide may retain their hydrolytic ability and facilitate solubilization of fat by lower ion concentrations, preventing or delaying micellar aggregation and precipitation. Hence, we theorized very low concentrations of hydroxide might provide adequate corrosion and also prevent the development of the semi-soluble material, particularly in the case of tissue with a significant amount of fatty material. To our knowledge the use of 1% to 5% hydroxide has not been reported in the literature. Therefore, we evaluated various concentrations of hydroxide corroding solutions using a quantitative filtration methodology. We corroded vascular casts from mice to test the practical effectiveness of corrosive solutions, given the limitations imposed by the dense networks of cast vessels.

Materials and Methods

Three different tissues were corroded to model a variety of animal tissues. Fatty tissue was chosen to corrode since it is the most time consuming to corrode. Chicken liver is a combination of fatty and protein rich tissue. Bovine tracheal rings are a tough, collagen rich connective tissue. Fatty tissue experiments utilized beef suet. Approximately 0.3 gram of suet was placed in 15 ml of each of the various corroding solutions. Sodium hydroxide or potassium hydroxide was added to scintillation vials and warmed to 45°C the day before adding tissue. Tissue was weighed and added to each corroding solution. After 8, 24, or 48 hours of corrosion, samples were filtered through a pre-weighed piece of Whatman number 1 qualitative filter paper (11 μm particle retention) using a suction funnel. Sixty ml of warm (45-50°C) water was used to rinse the vial used for corrosion and the sides of the filter funnel. The filter paper and retained material were air dried for 24 to 48 hours at room temperature and then weighed. Results were plotted as percentage weight change with time. All calculations were based on dry weights. Starting "wet" weights were converted to "dry" weights by multiplying with a correction factor for each tissue. Correction factors were determined after drying 5 samples of each of the three tissues for 48 hours and calculating the percentage of dry tissue. There was less than 1% change when tissue was dried for 24 hours or for 5 days. The average percentage of dry weight of beef suet after 24 or 48 hours was 96%. The dry weight fraction of chicken liver was 24% and the dry weight of bovine trachea was 36% of the initial weight. Each of the tissue samples was corroded in triplicate. Chicken liver and bovine trachea were obtained fresh from local retail or wholesale meat processing facilities. Procedures employed for liver and trachea corrosion were identical to those used for fatty tissue corrosion.

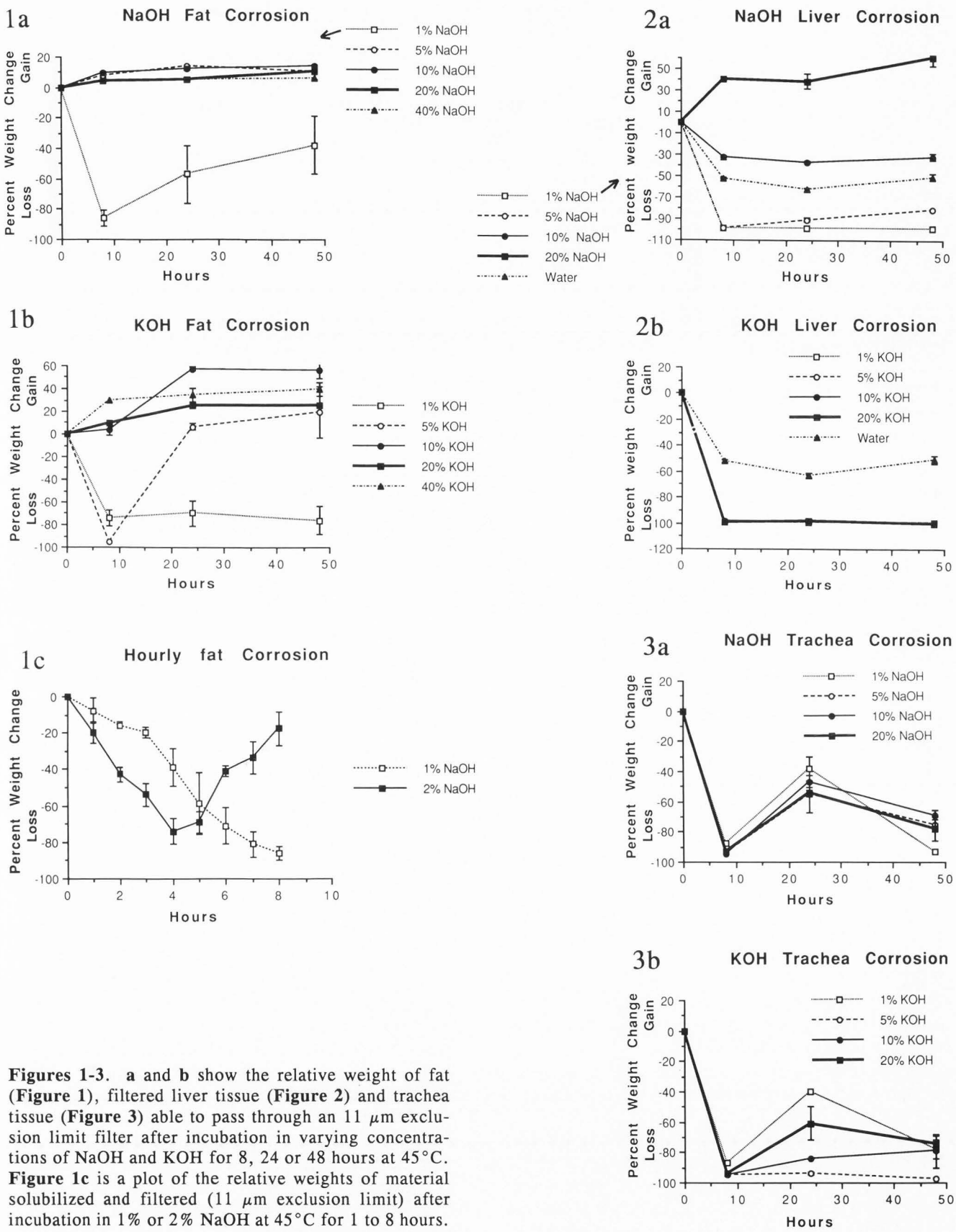
For evaluation of detergents, fatty tissue was corroded in different concentrations of NaOH (0, 1%, 2%, 5%, 10%, 20%, 40%) in combination with 1 percent detergent [(volume/volume, v/v) Triton-X-100 (Sigma), or (v/v) 7X (ICN Biomedicals) or (weight/volume, w/v) Terg-A-Zyme (Alconox)]. After being corroded 8 hours at 45°C, samples were filtered and dried as described above. Results were plotted as percentage weight change.

For tissue corrosion of cast organs, inbred Balb/c mice were cast *in situ* with methacrylate resin as described in Steeber and Albrecht (1992). After curing the resin for 24 hours at room temperature, tissue to be corroded was removed and placed in a 100 ml beaker with 50 ml of the appropriate corroding solution. After 8 hours at 45°C, casts were rinsed three times with warm tap water (45-50°C), rinsed three times in 100% ethanol (5 minutes each change), and air dried. After mounting and sputter coating with 30 nm of gold, vascular casts were viewed in a Hitachi S-570 scanning electron microscope operated at an accelerating voltage of 10 kV.

Results

Figure 1a shows the results for NaOH solubilized fatty tissue. Higher concentrations, 5% to 40% NaOH, show only a slight solubilization as measured by percentage weight change; also, there is little variation between different concentrations. A secondary increase in filter retained material with 1% NaOH and fat samples occurs after 24 and 48 hours. Figure 1c illustrates the solubilization of fatty tissue in 2% NaOH for the first 4 hours of corrosion then a decrease in solubility after 4 hours, as measured by an increase in filter retained material. As seen in Figure 1b, 5% KOH was the most effective in solubilizing fat in 8 hours. However, there is an increase in filter retained material at 24 and 48 hours of corrosion. One percent KOH does not cause the same increase in filter retained material as seen with the NaOH, as the potassium salt formed is more soluble than the sodium salt formed with NaOH as the corroding agent. The KOH filter retained material appears as a soft, translucent gelatinous mass that can be rinsed away with increasing amounts of water. The NaOH precipitate forms a firm, white mass apparently insoluble in water. Corrosion of liver (Fig. 2a) for eight hours is optimized in either 1% or 5% NaOH. After 24 hours, there is a decrease in solubility of liver in the 5% NaOH but not the 1% NaOH. Initially, water alone appears more effective in solubilizing liver tissue than either 10 or 20% NaOH. In contrast to NaOH, all concentrations of KOH were equally effective (Fig. 2b), and equivalent to 1% NaOH in solubilizing liver. No decreased solubility is observed over 48 hours with KOH.

The solubilization of collagen rich bovine trachea



Figures 1-3. **a** and **b** show the relative weight of fat (**Figure 1**), filtered liver tissue (**Figure 2**) and trachea tissue (**Figure 3**) able to pass through an 11 μm exclusion limit filter after incubation in varying concentrations of NaOH and KOH for 8, 24 or 48 hours at 45°C. **Figure 1c** is a plot of the relative weights of material solubilized and filtered (11 μm exclusion limit) after incubation in 1% or 2% NaOH at 45°C for 1 to 8 hours.

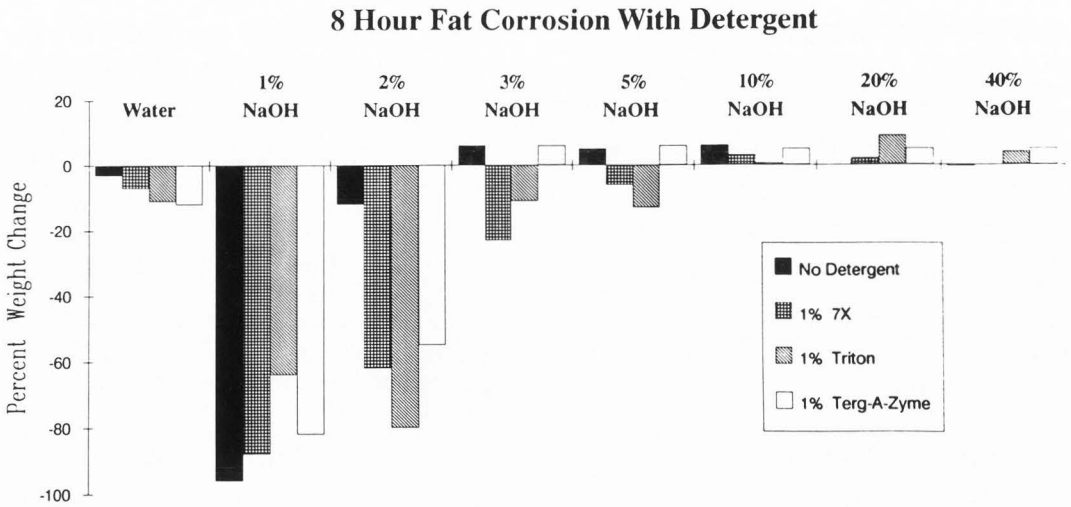


Figure 4. Effect of different detergents in the solubilization of fat in different concentrations of NaOH. Relative weight of fat able to pass through a filter (11 μm exclusion limit) is plotted.

showed relatively little difference between different concentrations of NaOH (Fig. 3a). Figure 3b illustrates the solubilization of trachea in KOH. An increase in retained material at the 24 hour time point is observed with the 1%, 10% and 20% KOH, but not the 5% KOH. After 8 hours of corrosion, 5%, 10% and 20% hydroxide (both NaOH and KOH) are equally effective.

Figure 4 summarizes results of the combination of detergent with different concentrations of NaOH in the corrosion of fatty tissue. Use of 1% NaOH alone is more effective without the addition of any of the three detergents tested in the solubilization of fat. The fat solubilizing action of either water, 2%, 3% or 5% NaOH was increased by the addition of detergent.

Mouse kidneys were cast with Mercox-methylmethacrylate resin to test the corroding effectiveness of 1% NaOH versus 15% KOH, given the physical restrictions to mixing and diffusion imposed by a dense network of cast vessels. Figure 5a is a micrograph of a glomerulus corroded 8 hours at 45°C in 1% NaOH, while Fig. 5b presents a glomerulus corroded for the same amount of time (8 hours) and at the same temperature in 15% KOH. The cast corroded in the 1% NaOH appears equally as clean as the casts corroded in the 15% KOH.

Discussion

With most tissues incubated in 1-5% hydroxide, solubility was greatest just after eight hours at 45°C. Corrosion of fat and liver longer than eight hours, particularly with hydroxide concentrations of 2% and above, can result in an increase in semi-soluble or insoluble products (filter retained material). The formation of reduced solubility products over time is attributed to the aggregation of micelles which are formed during the hydrolysis and saponification of the various tissues. Figure 1c illustrates the hydrolysis, saponification and production of micelles in the first 4 hours with the 2% NaOH and over 8 hours with the 1% NaOH. After 4 hours in 2% NaOH, reduction of charge boundaries by positively charged ions cause micelle aggregation of the colloidal suspension (sol), producing an increase in filter retained material and a resultant decrease in percent weight change. High ionic strength in 10 to 40% NaOH is believed to cause nearly immediate aggregation of micellar structures, rapidly producing a white insoluble product without the 4 to 8 hour delay as described above for the 2% and 1% NaOH respectively. The increased solubility of potassium salts of fatty acids accounts for the higher percent weight loss of fatty tissue using 1% KOH compared to the 1% NaOH after 48 hours. At the 8 hour time point, 5% KOH has solubilized over 95% of the fat compared to the 5% NaOH which has had a 5-10% weight gain. Low concentrations of hydroxides are effective for two reasons. First, the solubility of tissue is greater in lower concentrations of hydroxide. Secondly, colloidal suspensions produced (micelles) are held in suspension by "like" negative charges. The presence of positively charged sodium ions destabilizes or aggregates the colloidal particles. High ionic strength of hydroxide greater than 2%-5% may facilitate aggregation of micelles. At higher concentrations, saponification and aggregation occur rapidly. However, at lower (1% and 2%) concentrations, saponification and micellar formation

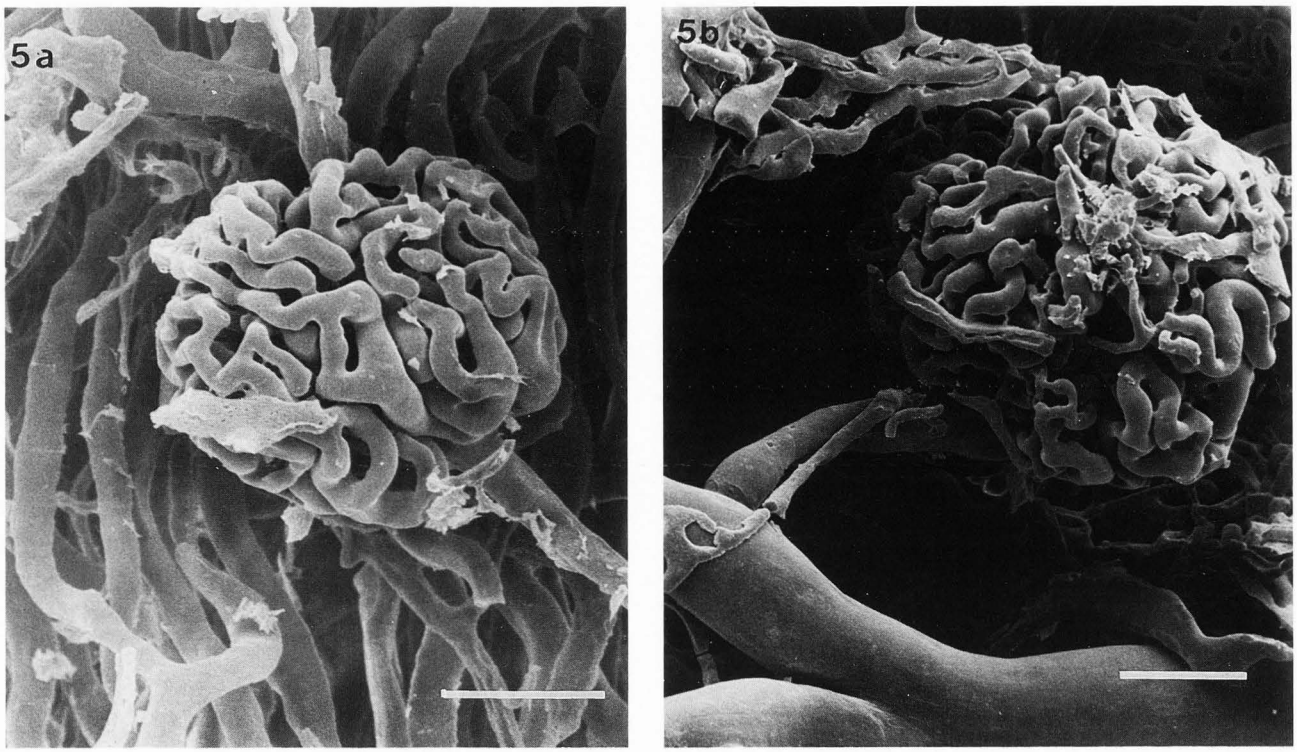


Figure 5. Micrographs from vascular casts obtained by corroding tissue in 1% NaOH for 8 hours at 45°C (**Fig. 5a**) and in 15% KOH for 8 hours at 45°C (**Fig. 5b**). Bar = 50 μm.

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occurs first, and subsequent aggregation does not occur until after 8 hours in the 1% NaOH or after 4 hours in the 2% NaOH. The addition of detergents to 2%, 3% and 5% NaOH, but not to the 1%, slows the saponification of fat and reduces the formation of insoluble products.

Low concentrations of hydroxides, particularly 1% to 5%, are as effective or more effective than higher, 10% to 40% hydroxides, in the solubilization of all three tissues evaluated. Corrosion of vascular casts in 1% hydroxides (NaOH or KOH) or 5% KOH appears effective in solubilizing and facilitating removal of tissue. The notion that the most rapid corrosion of tissue is obtained in relatively strong concentrations of hydroxide, as long as they do not harm the resin, appears generally not to be valid, particularly where any amount of fatty tissue is involved. Corroding solutions should be made up in distilled, deionized water, minimizing aggregation inducing cations. Both the time and temperature are important variables in the corrosion of tissue. Since corrosion is rapid and insoluble precipitates can form over longer time periods, we recommend changing corroding solutions after the first 6 to 8 hours and every 24 hours afterwards. Times and methods will vary with the type of tissue and the use of fixatives. Our experience has been that most unfixed tissues are cleaned in 8 to 48 hours in 1% NaOH. There appears to be no advantage to the use of hydroxides greater than 5% in concentration. The addition of detergents was relatively helpful in solubilization of fat in water, 2%, 3%, and 5% NaOH; however, solubilization of fatty tissue is greatest in 1% NaOH alone.

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Discussion with Reviewers

S. Aharinejad: Why did you analyze fat, trachea and liver obtained from bovine and avian sources for tissue digestion experiments, whereas corrosion casts were made from mouse kidney? How reliable is it to compare tissue digestion to casts made from different organs and different species?

Authors: We chose bovine trachea, and fat and chicken liver, for convenience and as clear examples of different tissue types as described in the text. Although some differences in corrosion of a given tissue from different species may exist, generally our experience with hydroxide corrosion has been that similar types of tissue corrode in a similar fashion regardless of the species of origin. This is probably a function of the chemistry involved, which is not particularly sensitive to minor changes in composition.

M.A. Konerding: Please comment on the possible effects of fixatives on the maceration process?

Authors: Fixatives can have a pronounced effect on corrosion rates. After 24 hours at 45°C, mouse kidney and liver, perfusion fixed with 1% glutaraldehyde and 3% formaldehyde, appeared to corroded most rapidly in 20% KOH as compared to 1%, 5% and 10% KOH. After 48 hours, 5%, 10% and 20% KOH were equally effective, while the 1% KOH solubilized about one third of the kidney and liver. Thus non-fatty, fixed tissue appears to corrode more rapidly in the higher concentrations of KOH. Corrosion in NaOH was not evaluated.

D.E. Schraufnagel: In the corrosion study I carried out (Schraufnagel, 1989) I was disappointed that there was not a greater difference between the various conditions affected casting, but I used concentrated hydroxide. Do you think the effect of pre-alkali autolysis, detergent and proteolytic enzymes were blunted by too concentrated hydroxide?

Authors: The effect of pretreatment probably was blunted by the high concentrations of hydroxide, however, we feel the use of pre-alkali treatments probably have a less significant effect than the use of low concentrations of alkali alone.

D.E. Schraufnagel: I am surprised that your casts shown in Figure 5 are so free of tissue in just 8 hours. Do you think the minor debris that remains would be removed by rinsing only or is further corrosion required? For how long is final rinsing necessary?

Authors: We change to fresh corroding solution generally after 6 to 8 hours of corrosion and then every

24 hours afterwards. When the corroding solution remains clear for 24 hours, if possible, we view a small piece of dried cast with a dissecting microscope. If the cast appears free of tissue, we then rinse with distilled water for 10 to 20 minutes before drying.

D.E. Schraufnagel: Has multivariate statistics been performed on the data to confirm which differences are important?
Authors: Analysis of variance at p value less than or equal to 0.05 confirmed the following results: For corrosion of fatty tissue, 5% KOH was better than 1% KOH or 1% NaOH. Liver corrosion after 8 hours was not statistically different among 1%, 5%, 10%, 20% KOH, and 1% and 5% NaOH. Trachea corroded equally in 5%, 10% or 20% hydroxide (KOH or NaOH) after 8 hours.

S. Aharinejad: Do you think that endothelial cells could be mummified during the casting procedure and in this way withstand the maceration? Accepting that endothelial cells, or any other perivascular cells or tissue components could be mummified (Aharinejad and Böck, 1993) by casting medium and become resistant against corrosion; how could we reliably distinguish a sufficiently macerated cast with mummified cell on its surface from a cast which may need a longer digestion of remaining soft tissue?
Authors: We do not have experience with mummified cells, although we would expect mummified cells to be resistant to maceration. We do not know of a reliable way to distinguish when a cast is completely macerated other than to prepare and observe a small portion of the sample by light and scanning electron microscopy. Our experience with unfixed tissue is that a maximum of 5 days should be sufficient to clear any cast when starting in 5% KOH at 45°C for 6 to 8 hours and switching to 1% KOH (45°C) changed every 24 hours. Your 1993 paper contains more information regarding mummified cells.

S. Aharinejad: Would the dilution of Mercox with methacrylic acid have any influence of the corrosion resistance of obtained casts?

Authors: With increasing amounts of methacrylic acid monomer, there is the chance that not all the monomer will polymerize and therefore would be less resistant to corrosion. We are not aware of any difficulties with corrosion of casts diluting Mercox 1:1 with methacrylate monomer. In addition, the lower concentrations of hydroxide should be less harmful to the cast material.

A. Lametschwandtner: Could you please give a recommendation for the most effective maceration solution for brain tissue?
Authors: We would recommend 5% KOH prewarmed to 45°C. Rinse well after 6 to 8 hours and place in fresh 1% KOH at 45°C, change every 24 hours if continued maceration is necessary.

S. Aharinejad: Did you examine the vascular cast's surface (e.g., endothelial cell nuclei imprints, endothelial cell border lines), after applying your tissue digestion procedure, in scanning electron microscope?
Authors: Qualitatively, we see no differences resulting from corrosion at one concentration of hydroxide versus another, provided all biological tissue has been removed.

S. Aharinejad: We know (just empirically) that tempering facilitates the tissue digestion. Why?
Authors: The increase in reaction rates and the increased solubility of solutes are probably the major factors facilitating tissue solubilization. Generally, we corrode tissue at 45°C, although Weiger *et al.* (1982) indicated the embedding resins Mercox and methylmethacrylate (MMA) to be stable up to 33°C and 44°C respectively. They also noted (Weiger *et al.*, 1986) that tempering of Mercox and MMA mixtures at 60°C for 12 hours improved the thermostability of the resultant casts. The optimum corrosion temperature for each resin or combinations of resins has yet to be accurately determined. Our experience has been that tissue corrosion with 1-5% hydroxide remains effective at 37°C (and probably lower temperatures), so that corrosion at lower temperatures to maintain cast stability poses no problems.