

DEHYDRATION OF MACROSCOPIC SPECIMENS
BY FREEZE SUBSTITUTION IN ACETONE

Klaus Tiedemann and Dubravka Ivic-Matijas

Anatomical Institute I
University of Heidelberg
Im Neuenheimer Feld 307
D-6900 Heidelberg
Federal Republic of Germany

INTRODUCTION

Dehydration of bulky specimens has attracted little research interest and has been performed mainly with a graded series of alcohols. Dehydration by freeze-substitution in acetone is recommended for plastination (1)(2), a method for preserving biological material by impregnation with curable polymer. In this process, the acetone also accomplishes defatting and serves as an intermediary solvent.

Freeze-substitution is based on the same principle as cryosubstitution, a dehydration procedure used in histochemistry and electron microscopy (3). Tissue to be dehydrated is removed from the fixative and placed in -25°C acetone. Water within the specimen freezes immediately upon immersion, providing an internal scaffold that tends to hold its shape and dimension. Slow substitution of ice by acetone then results in only minor shrinkage. Crystal formation will distort microscopic morphology, however, if the specimen is larger than 2 mm.

The few protocols published for freeze-substitution of whole organs recommend a minimum of three changes of an acetone bath, having a volume ten times that of the specimen. Dehydration time of up to five or six weeks is usually stipulated (4)(5). The present investigation was performed to arrive at a procedure that was more economical in both time and solvent. Water exchange was monitored daily in an effort to determine how many changes of what volume and grade of acetone is required to reach the minimum time of exposure for complete dehydration.

MATERIALS AND METHODS

All specimens were human tissue. Bone with attached muscle, uterus with bladder and rectum, prostate with bladder and rectum, tongue with larynx, as well as brain halves, were used. Most of these organs had been stored in a freezer prior to fixation and dissection. All were fixed in formalin, rinsed with tap water, pre-cooled to $+5^{\circ}\text{C}$ and blotted prior to immersion in acetone at -25°C .

Ten fixed hearts with epoxy-injected coronary arteries were exposed to -25°C acetone under hydrostatic pressure (via cannulated vessels). This was done in an effort to freeze and partially dehydrate them in an expanded state. These hearts were then used as part of this investigation. As expected, they showed a lower amount of water extraction during immersion, however they were considered valuable because they were easily monitored for shrinkage artifact.

A total of more than 55 specimens were used in 22 separate dehydration exercises. In most of these, two similar organs were weighed, placed in closed containers, filled with acetone at -22 to -25°C and maintained at this temperature for the duration of the procedure. Technical-grade acetone (99.9% by volume) was used throughout. For convenience, this is listed as 99% in all graphs. All unspecified solvent changes refer to this almost-absolute grade of acetone. Each bath consisted of 2 to 5 liters of acetone. This amounted to 5 to 12 times the sample volume. Both organs in any one series were treated in an identical

manner. In experiments that started with dilute acetone, 99% solvent was purposely diluted; used acetone was not employed.

Organs were turned every 24 hours, at which time hollow specimens were emptied and refilled. In calculating the percentage of water removed, the acetone concentration of a bath was measured, using a densitometric spindle graded in 1/10 degrees. In some cases, water determination was performed using the Karl Fischer, titrimetric method. After freeze-substitution, specimen containers were transferred to a cold room at +5°C for one or two days and then removed to ambient temperature.

RESULTS

The water content of fresh tissue is known from the literature. To compare this value to that of the formalin-fixed tissue used in these experiments, we allowed samples of fixed tissue used in pilot experiments to dry up, weighing them before and after. By this method, samples of gut (with mesentery) lost 72% water. Brain, 73-75%, embalmed liver, 74% and liver fixed via the portal vein, 89%.

Shrinkage of 7mm brain slices was determined as a result of exposure to an initial bath of 60%, 70%, 80%, 90% and 95% cold acetone (-25°C). It was observed that concentrations of 60 to 80 percent do not lead to shrinkage. In these dilute solvents, a swelling appeared during the first few days, expanding 100 mm slices to 101, 102 or 103 mm. After 4-8 days, this swelling receded to almost the original dimension. In 90 and 95% cold acetone (-25°C), this swelling was less apparent or absent. After 9 days, 100 mm slices measured 97.5, 99, 100 and 101 mm.

The rate of penetration of -22°C acetone into 4 cm tissue cubes was checked using hard, fixed liver from the dissection laboratory and softer, fresh-frozen liver. The depth of penetration of 70% acetone was no more than 3 mm after 24 hours and only 4-8 mm after 9

days (in regions devoid of large vessels). The core of all specimens was still frozen. The serosal surface acted as a diffusion barrier. The softer sample was penetrated slightly more rapidly than the harder by this concentration of acetone.

In 98% acetone, both kinds of tissue were penetrated faster. One day resulted in 5 mm penetration into the soft block and 4 mm into the hard block. The frozen core of the soft block had been replaced after 4 days. The hard block was penetrated 10 mm in 4 days and its ice core was negligible after eight days.

The tissue-to-solvent volume ratio was of no critical importance. The recommended ratio of 1:10 was found unnecessary. Experiments showed that a ratio of 1:5 is sufficient (Fig. 5) and that, at this ratio, the first bath equilibrates at about 12% water. Thus, doubling the volume of specimens in a dehydration bath would be the first means of reducing expense.

The gravity-induced concentration gradient in deep cylinders filled with old acetone is remarkable. At the bottom, the water content may be as much as 3.5% higher than at the surface. Specimens must be kept off the bottom by suspension or elevation on a grid.

Graphs provide an interesting means of following a dehydration experiment. For example, it can be seen quite clearly that time is wasted by waiting too long before an acetone change. Once the water content of the bath equilibrates with that of the specimens, no further exchange of water takes place (Figs 2,5). Acetone change during a steep part of the curve, on the other hand, would lead to a waste of solvent since the potential for exchange into the old bath will not have been fully utilized.

Our first experiments showed that rewarming is the most critical factor with respect to tissue shrinkage. If tissue, still containing more than 10% water, is moved from -22°C

to room temperature, it will suffer severe shrinkage because 5-8% of its original water will be extracted in one day. We therefore introduced a +5° step (dashed line on graph) between the freezer phase (solid line) and ambient temperature (dotted line). Changes into fresh acetone at +5°C were not performed before the container had assumed the same temperature. Circles, triangles and squares that are filled in indicate not only a measured value but also a change of acetone.

Figure 1 demonstrates that similar organs, dehydrated under similar conditions, will manifest approximately the same dehydration curve. Further, the reproducibility of these results is rather high.

In the experiment shown in Figure 1, dehydration of a heart in a short period of time with only a small amount of shrinkage was achieved, but at the expense of 25 liters of acetone for only 500 grams of tissue. The first acetone bath (up to the end of the second day) removed approximately 35% of the organ's weight as water. The first acetone change in the heart represented by circles was made too early (on the steep part of the slope). Both acetone changes after three days came too early and the second bath removed far too little water. The third bath also was inefficient.

The heart experiment shown in Figure 2 was performed with a graded series of cold acetone. More appropriate timing of changes was conservative of solvent and only three baths were employed. The heart that was started in 80% acetone (circle) showed no further water extraction in this first bath during the second day. The heart started in 90% acetone (triangle) still lost 9% water during the second day. On days two and four, the inclination of the curve indicates that it would have been more economical to change the solvent about one day later. Gradual rewarming of the specimens at this stage, even without an acetone change, enhances dehydration but still holds shrinkage to a minimum. Since the curve of the specimen represented by a circle is not yet

horizontal by day 9, a third change, into a smaller acetone volume, would have been necessary to get below 1% residual water.

As a general rule, the proper use of a graded dilution series of cold acetone extracts more water than starting with pure acetone (Figs 2, 3, 5, 6). Figure 3 shows that the third day is most advantageous for the first change. The second change should be two or three days later (Figs 3, 4). Warming the specimen to +5° should take one or two days (Figs 4, 5, 6). The second day at this temperature without an acetone change (Figs 3, 5) removes less than 2.5% water. The rate of water extraction will, of course, be influenced by the shape and consistency of the specimen. In Figures 4 and 5, for example, pelvic organs show a large percentage of water (up to 60%) extracted in the first bath.

Dehydration of bulky specimens, in which water and solvent exchange must take place through a considerable mass of tissue, requires much more time than the process described above. Brain halves with water trapped below the meningeal membranes (Fig 6) required 24 days in cold acetone for near-complete extraction. This resulted in shrinkage of 2%. The many acetone changes certainly were not necessary. The ice core must have remained intact for more than one week. The sudden onset of shrinkage (5.2-6%) coincides with the specimens' being warmed to ambient temperature, at which time fat extraction begins. This can be avoided and will be discussed in a separate paper.

DISCUSSION

The purpose of this investigation was to establish a dehydration procedure that was most conservative of solvent and time but still resulted in minimal dimensional change and adequate dehydration. Water-extraction characteristics of various organs were examined and several principles defined. These should be considered recommendations rather than inviolable rules, however,

These should be considered recommendations rather than inviolable rules, however, because certain inherent limitations of measurement precluded complete accuracy.

One such measurement difficulty centers around determination of water actually extracted. For example, it is assumed that the amount of water leaving the specimen equals that of the acetone entering. In fact, the total specimen volume diminishes by about 5% during the first days and increases again later in the process. Also, use of the densitometric spindle is very temperature - dependent, as well as sensitive to the lipid content of the acetone bath. And the Karl Fischer titrimetric method is not absolutely reliable in the presence of 20-30% water. Although exact determinations of water extraction and shrinkage can be done on small, homogeneous tissue cubes (6), these will not accurately predict changes occurring in large specimens during this same process.

Using freeze-substitution, shrinkage can be kept below 10% (7), much less than that experienced with a graded series of alcohols at room temperature and quite acceptable for macroscopic specimens. In systematic experiments intended to define shrinkage (8), temperatures between -28 and -30°C were found optimal. Brain shrinkage increased noticeably at higher temperatures, beginning as low as -25°C.

Gradual rewarming is absolutely required for control of specimen dimension. Abrupt warming from -25°C to room temperature can lead to massive shrinkage from sudden water extraction. Thus, the dehydrated specimen should always be kept for one or two days at +5°C to permit slow water equilibration at this intermediate temperature before restoring it to ambient temperature.

The rate of p on large liver cubes and brain halves during freeze-substitution (mentioned above). Thus, when working with thick, bulky specimens, an acetone change after three days may be too early since penetration has been too sluggish to reach the central ice core. This notwithstanding, even large

specimens (subjected to three bath changes) will have reached near-complete dehydration within 9 to 11 days, after which the final bath will not contain more than 1% water.

It seems, then, that dehydration time can be significantly reduced from the six weeks recommended in an earlier publication (10). Three changes of solvent remains the rule and an intermediate warming to +5°C should be considered essential. Given a tissue-acetone volume ratio of 1-5 and these revised recommendations regarding exposure time, acetone consumption can be safely reduced to almost one half and time to about one third when compared to previously accepted minimum standards (10X11).

An additional economy can be realized by re-using acetone. Although used acetone causes no damage, once it becomes diluted to 70% it is ineffective, except as a first bath. Based on our experience, we feel that the previously recommended dilution limit of 95% for an initial bath (11) is far to high. Cost can be further reduced if the final bath from a previous dehydration is re-used as a second bath.

REFERENCES

1. Bickley, HC ; Donner, RC ; Walker, AN ; Jackson, RL
Preservation of tissue by silicone rubber impregnation
J Int. Soc. Plastination 1:30-38, 1987.
2. Bullivant, J
Freeze-substitution and supporting techniques
Lab. Invest 4:440-457, 1965.
3. v Hagens, G ; Tiedemann, K ; Kriz, W
The current potential of plastination
Anat. Embryol
175:411-421, 1987.
4. v Hagens, G ; Schwab K
In preparation.

5. Lischka, M ; Prohoda, M
Plastination of whole-body slices with
polymerizing emulsion
J.Int. Soc. Plastination
1:17-22, 1987.
6. Sasse, D ; Matthaei C
Improvement of freeze- substitution by
programmed rewarming
Stain Technol. 52:299-300, 1977.
7. Schwab, K ; v Hagens, G
Freeze substitution of
macroscopic
specimens for plastination
Acta Anat. 111:139-140, 1981.
8. Seki, M
Untersuchungen mit nichtwässrigen
Flüssigkeiten.
1. Fixierung Gewebe in
nichtwässrigen Flüssigkeiten.
Z. Zellforsch.
26:305-320, 1936
9. Sitte, H ; Neumann, K ; Edelmann, L
Cryofixation and cryosubstitution for
routine work in transmission electron
microscopy.
In: Muller, MR ; Becker, B ; Boyde,
A; Wolosewick, JJ (eds.)
The science of biological specimen
preparation.
SEM Inc., AMF O'Hare, IL 60666,
103-118, 1986
10. Tiedemann, K ; v Hagens, G ;
The technique of heart plastination
Anat. Rec.
204:295-299, 1982

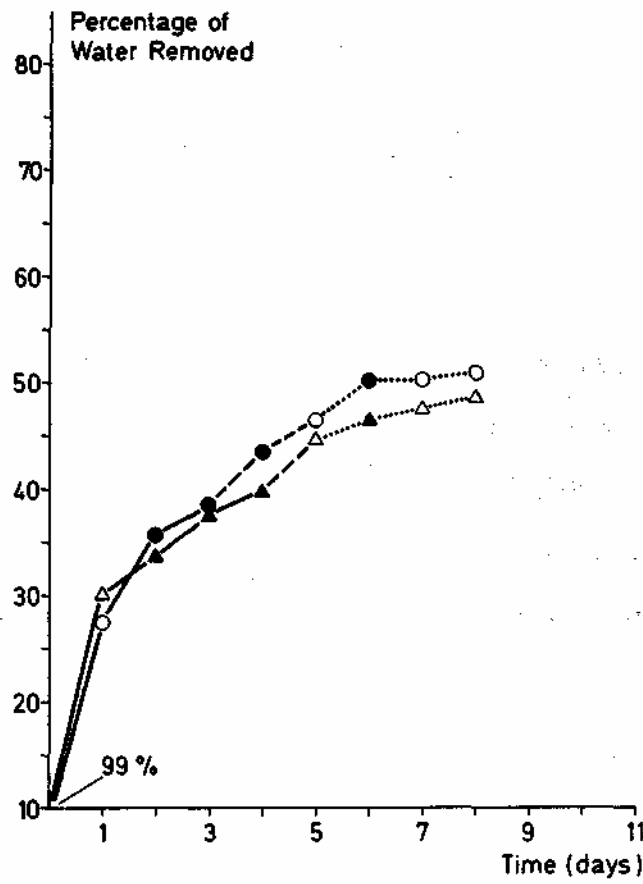


Figure 1

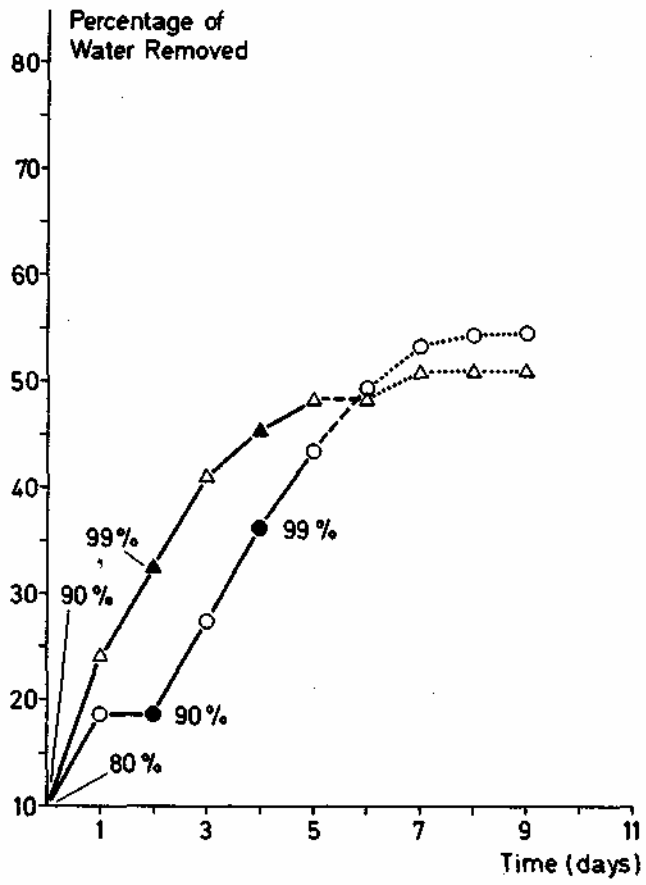


Figure 2

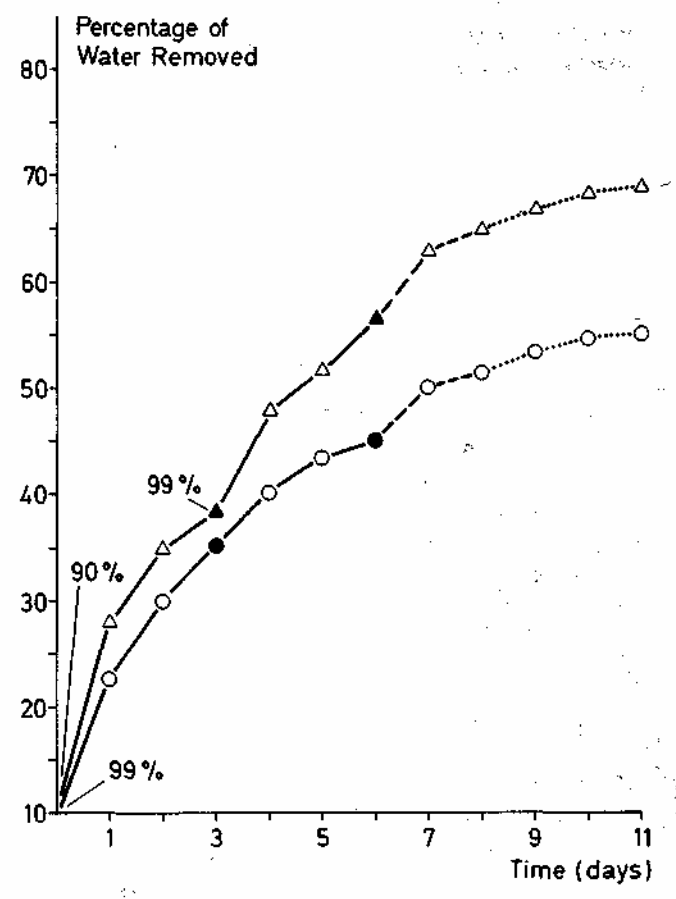


Figure 3

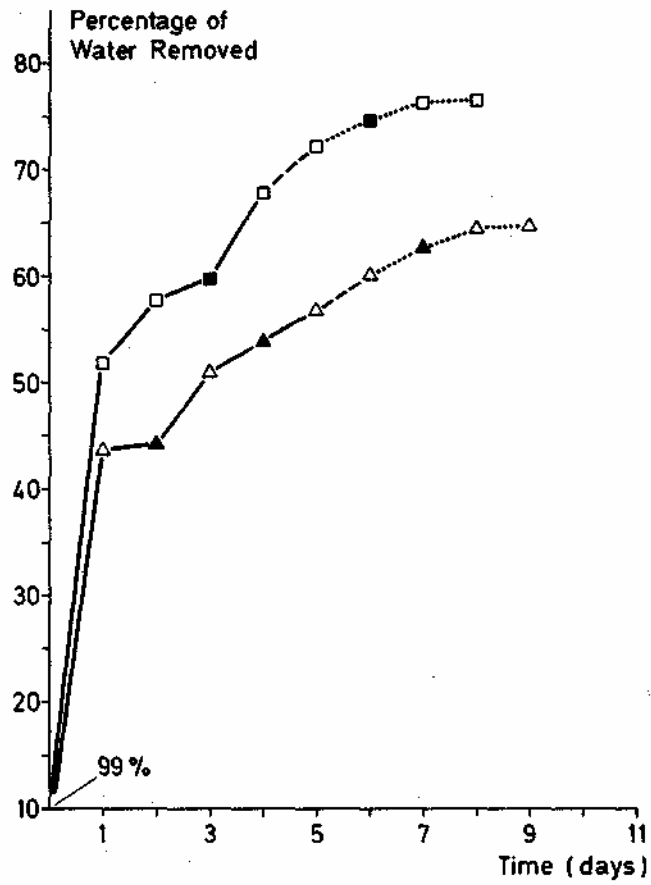


Figure 4

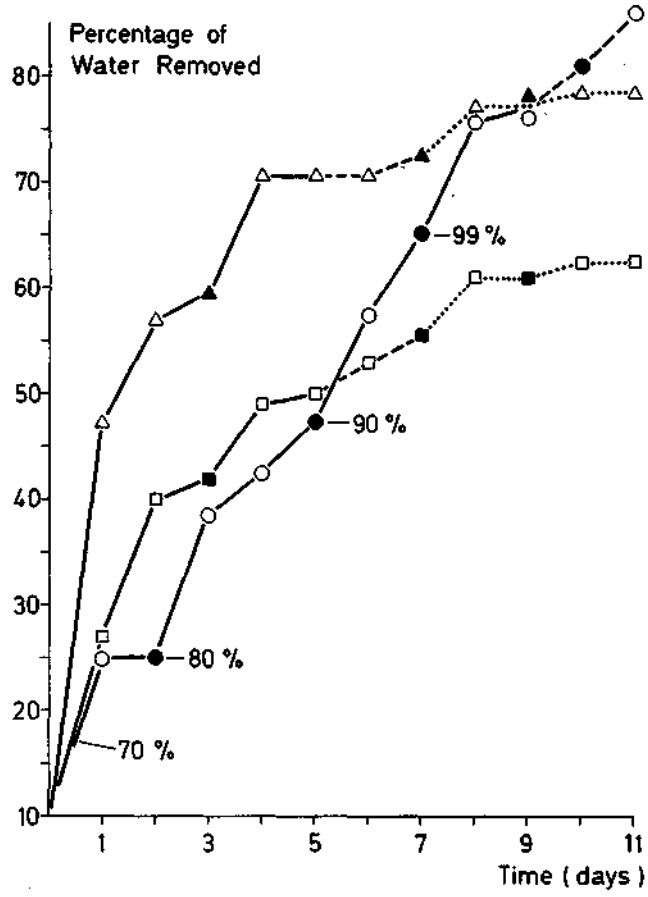


Figure 5

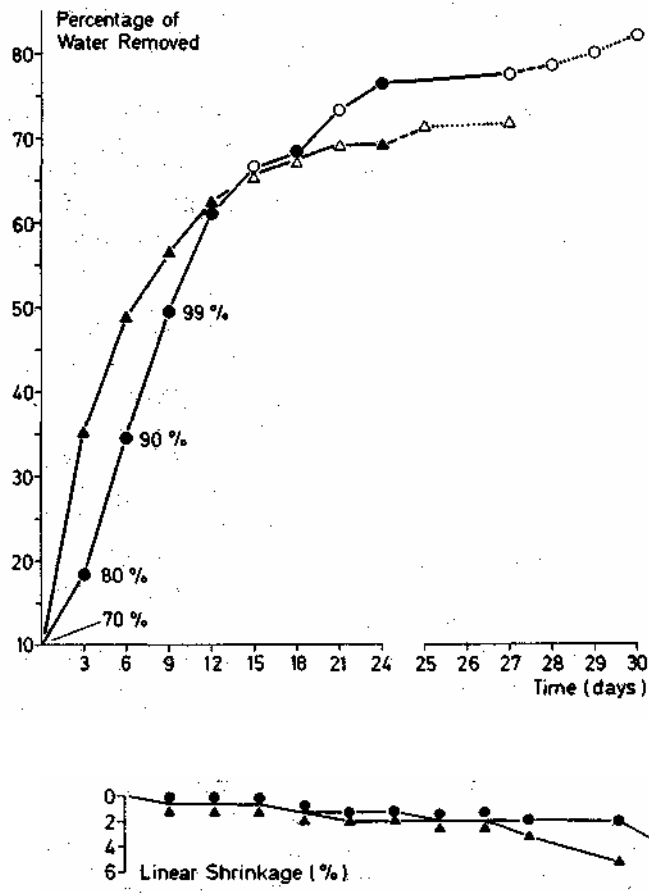


Figure 6