DRAFT

Fixation of the Human Brain After Prolonged Postmortem Intervals as a Prelude to Cryopreservation

By Michael G. Darwin

UNDERSTANDING ALDEHYDE FIXATION

In order to understand the limits of immersion fixation on a tissue mass as large as the human brain it is first necessary to consider the actions of the aldehyde fixatives on tissues.

Factors affecting reproducibility

As long ago as the first half of the 20th century Underhill, et al., (1) reported that formaldehyde was a very slow fixative for tissues which validated the axiom that "*formaldehyde penetrates rapidly but fixes slowly*." This is, in fact, the central paradox of formaldehyde fixation and one that poses a serious problem to achieving reproduceable results in the laboratory. Another factor complicating the repeatability of the performance of formaldehyde as a fixative is the rapid but variable oxidation of formaldehyde to formic acid in the presence of atmospheric oxygen, which may occur either as a result of the manufacturing process, or as a result of storage on the shelf. An indication that this has occurred is the formation of "formalin pigment" when blood-containing tissues are fixed in formalin solutions. On subsequent microscopic examination, a finely divided birefringement pigment can be seen to have precipitated in the tissue, probably a consequence of hematin formed when the pH of the fixative is below 6.0

Formaldehyde, when dissolved in water, rapidly becomes hydrated to form methylene glycol. While the formation of this derivative was well known to Blum (2) in the early part of the last century, the interaction of methylene glycol with biological tissues has been surprisingly little studied by histologists and electron microscopists. The reactivity of

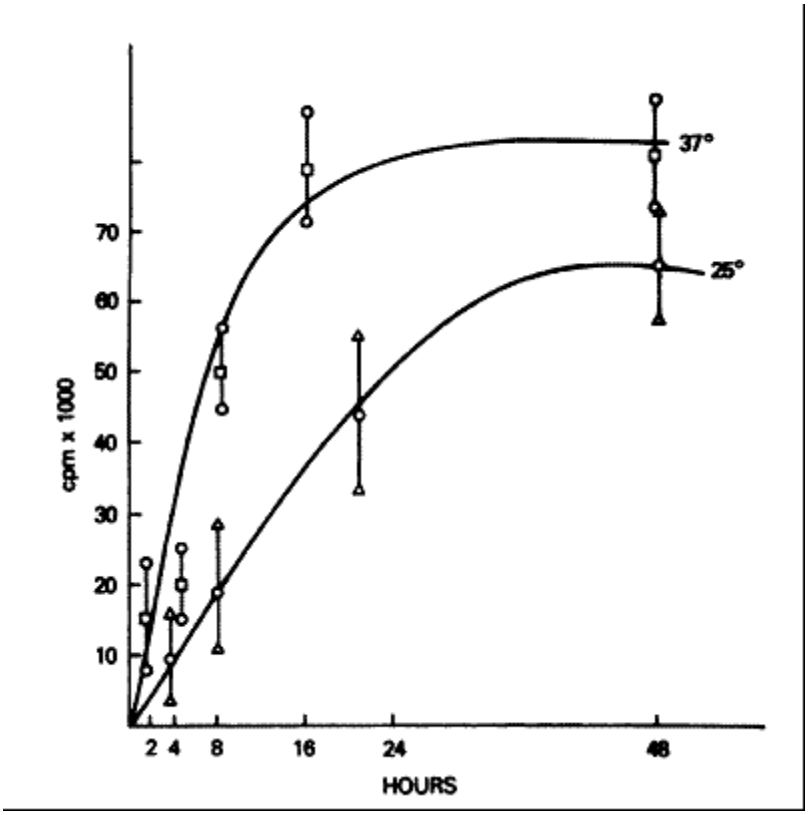
methylene glycol is known to physical chemists as an example of a "clock" reaction meaning that the equilibrium between methylene glycol and formaldehyde in aqueous solution so favors methylene glycol that the conversion of methylene glycol to formaldehyde by the removal of formaldehyde can be used as a "real-time" clock, measurable in hours (3). When tissues are immersed in methylene glycol solutions they are penetrated rapidly by methylene glycol and the fraction of formaldehyde present in the solution at the time.

Actual covalent chemical reaction of the fixative solution (e.g., that fraction of formaldehyde present) with the tissue *depends upon the formaldehyde present being consumed after forming bonds with the tissue components and more formaldehyde forming from dissociation of methylene glycol* (4). Leather chemists studying the tanning process who have long been interested in the reaction of formaldehyde with the collagen in hides, use conditions for tanning that favor the dissociation of methylene glycol, such as low pH and high concentrations of methylene glycol as well as elevated temperatures. Thus, equilibrium between formaldehyde, as carbonyl formaldehyde, and methylene glycol, explains most of the paradox of why formaldehyde (as methylene glycol) penetrates rapidly and fixes slowly (as carbonyl formaldehyde). This also explains the mystery of the inconsistent and often variable results seen in the histologists' and electron microscopists' laboratories with respect to fixation with formaldehyde in particular, and aldehydes more generally.

This is a very important point because it highlights that even very experienced academic and professional scientists' using well standardized methods not infrequently achieve very different outcomes with respect to fixation. Therefore, histology and electron microscopy are rightly considered, at least in part, an art as well as a science.

The "formaldehyde paradox" was first explained by M. G. Burnett in the early 1980s with the elucidation of its behavior according to the clock reaction (5). Further studies building on Medawar's original kinetic modeling of methylene glycol's diffusion kinetics into plasma clots by Baker (6) using blocks of gelatin-albumen gel to more closely approximate solid tissue, and the work of Tellyesniczky (7) who used blocks of rat liver tissue, determined a diffusion constant of K = 0.78

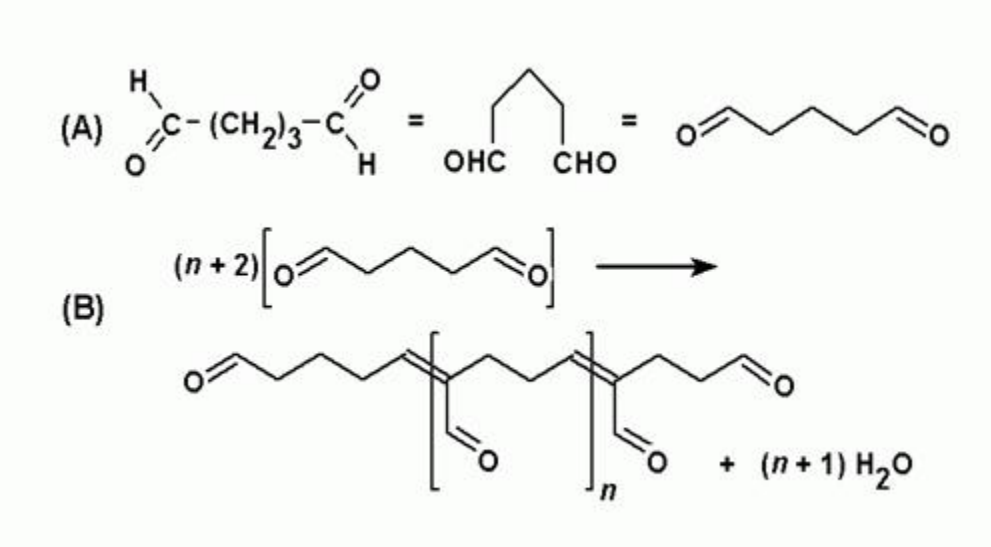
for methylene glycol. A diffusion constant of K=0.78 would imply that that the penetration rate of methylene glycol into tissue would be on the order of 3.9 mm in 25 hours at 25°C (8).



The covalent binding time of radiolabeled 14C formaldehyde at 25°C and 37°C are shown above. Equilibrium is not reached until 18 hours 37°C at and not until 24 hours at 25°C (8).

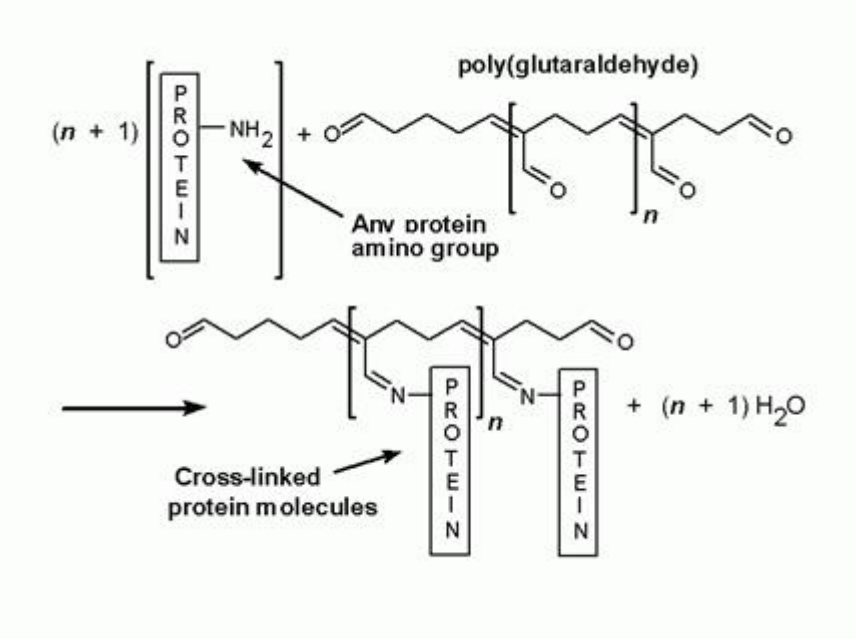
As can be seen in the graph above, the measured diffusion rate of radiolabeled 14C formaldehyde to empirically determine the covalent binding time for formaldehyde in rat renal cortex at room temperature (e.g., 25°C). They found the amount of formaldehyde bound to tissue increased with time until equilibrium was achieved at 24 hours. At 37°C the binding rate was considerably faster, and equilibrium was reached at 18 hours. A later study by Healander also employing 14C labeled formaldehyde, but employing rabbit liver rabbit liver rather than kidney sections, yielded similar results, with an equilibrium binding time of 25 hours at 25°C.

However, the chemistry and kinetics of glutaraldehyde fixation are even more problematic. Glutaraldehyde consists of two aldehyde groups connected by a flexible chain of 3 methylene bridges. Its molecular weight is 100.12 with the molecular formula being HCO-$(CH_2)_3$-CHO. As is apparent from its structure, it has a high propensity for cross-linkage via its -CHO groups, which can occur over variable distances:



Three representations of a molecule of monomeric glutaraldehyde. ***(B)*** *Polymerization reaction of glutaraldehyde, showing an aldehyde sidechain on each unit of the polymer.*

Thus, glutaraldehyde is present largely as polymers of variable chain lengths in aqueous solutions with a free aldehyde group protruding from the side chain of each polymer unit and at the end of each molecular unit (10). (see illustration above). All the -CHO groups are available to combine with any NH_3 groups on proteins with which they come into contact. Therefore, there is enormous potential for cross-linking:



Reaction of poly-glutaraldehyde with amino groups of proteins.

There are also many left-over aldehyde groups (not bound to anything) that cannot be washed out of the tissue.

When glutaraldehyde, a dialdehyde with a molecular weight three times that of formaldehyde, is used for fixation a much lower molar concentration is used. Glutaraldehyde has the advantage that most of the aldehyde groups in the solution are not bound up as glycols. A 3% solution of glutaraldehyde has a much lower osmolality than 4% formaldehyde, and yet it has significantly more available reactive aldehyde groups for fixation.

The downsides of glutaraldehyde as a fixative are its much more adverse diffusion kinetics and its vastly longer "clock time" reaction. Therefore, it is used as a "secondary" fixative rather than as the primary fixative.

If an ischemic brain is perfused with aldehyde fixatives it will, presumably as a result of the no-reflow phenomenon, experience both less uniform distribution and a much lower total (global) concentration of fixative. This will be especially true for glutaraldehyde which diffuses into the tissues much less rapidly than does methylene glycol. Indeed, the decrease in aldehyde, and in particular glutaraldehyde concentrations, may be proportionally

greater than in a freshly perfusion fixed brain because a significant fraction of the aldehyde may still be confined to the pial vessels on the surface of the brain not having had time to diffuse out into the tissues of the cerebral cortex. This is an important observation that takes on more significance later, in the discussion of brain angiography after long postmortem intervals (PMI) and its potential application to achieving adequate brain fixation under adverse circumstances.

FACTORS LIMITING THE STABILITY AND LONGEVITY OF ALDEHYDE FIXED TISSUES

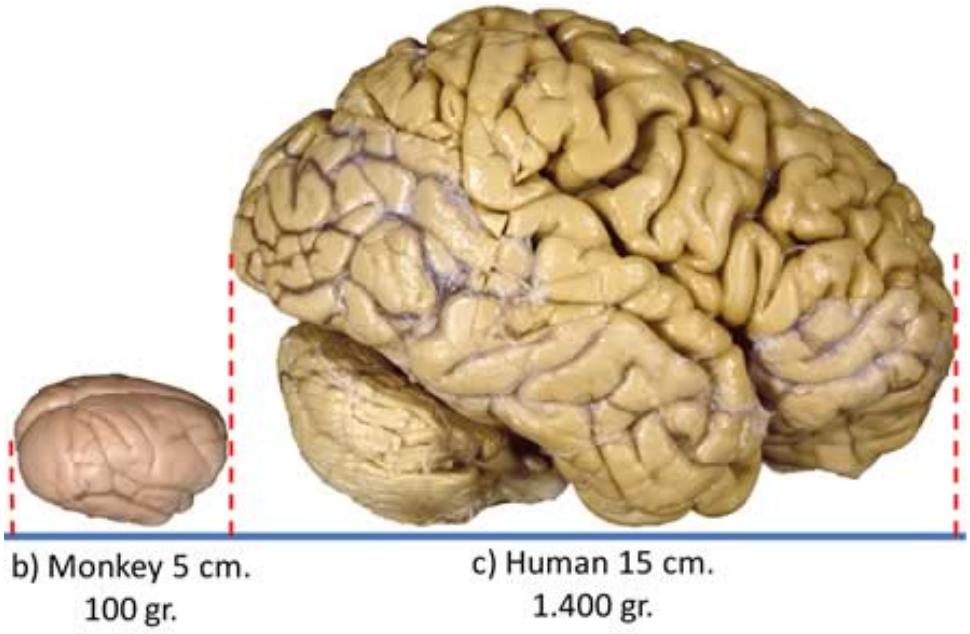
Much of the preservative effect of aldehydes is due not to their protein cross linking effects, but to their bacteriostatic and bactericidal effects. It is also the case that aldehyde fixation does nothing to stoop to halt oxidation of brain lipids and proteins as a result of the diffusion of ambient oxygen into the organ. Nor does it inhibit the hydrolysis of brain macromolecules as a result of the bombardment by energetically active and chemically reactive water molecules. Thus, the *fate of all aldehyde fixed tissues is complete decomposition*. Once the aldehyde concentration drops below the bacteriostatic threshold, microorganisms will begin to first metabolize the unfixed lipids and carbohydrates and will ultimately decompose even the most densely methylene bridge cross-linked proteins. For this reason, large tissue masses preserved in aldehydes should have fixative fluid replaced with freshly prepared fixative at ~2-year intervals.

HOW EFFECTIVE IS IMMERSION FIXATION OF THE BRAIN?

In the mid-1990s, Vertes and Crane demonstrated good preservation of neuronal connectivity in the Rhesus monkey brain using simple chilled immersion fixation (e.g., immersion of the un-perfused brain in chilled fixative immediately following its removal, followed by subsequent refrigeration at ~4°C (11-13).

This is perhaps credible because the of the large dimensional differences between the brain of a human and that of a rhesus monkey. The human brain is 4.8 times the size for a hypothetical monkey of the same body weight and the cortical surface area is ~11,000 mm^2, as opposed to ~92,400 mm^2 for that of the average human brain. At its thickest is the cerebral cortex is ~ 5 cm for a

rhesus monkey vs. ~11-15 cm for a human brain. The monkey brain can be expected to undergo complete fixation via immersion at 4°C at ~4-5 times that typically achieved in the human brain.



Very recently, due to the passage of laws in a number of states forbidding coroner's and medical examiners from retaining tissues from the deceased for any significant period of time, there has been research done to evaluate the effectiveness of rapid, often only overnight fixation, on the histological structure of human brains (14-16).

This work has shown that the cerebral cortex appears to be histologically, and perhaps ultrastructurally, well preserved by immersion fixation. Given that the average thickness of the human cerebral cortex is ~ 3 mm (17, 18).

Studies on the immunohistochemistry of immersion fixed rat and human brains also demonstrate a surprising degree of preservation at the biochemical level, as well (19,20).

MEASURED EFFECTIVENESS OF IMMERSION FIXATION IN HUMAN BRAINS

Thus, if the idea of the connectome as both necessary and sufficient to encode personal identity is considered valid, it could be argued that even prompt, postmortem *immersion fixation* may stabilize

enough brain structure to permit survival of the individual. The author is not currently of this opinion but the point here is that once you start down the rabbit role of solely, or mostly unproven, *theoretically based* preservation technologies for personal survival, well, you are pretty well lost (unless you make an extraordinary effort to continuously and rigorously generate feedback which is subsequently rapidly used to drive relentless improvement in the procedure).

However, if the criterion for fixation-preservation of the brain is adequate fixation of *more* than to a ~3 mm depth of the cerebral cortex, immersion fixation will not be adequate. If the foregoing theoretical and experimental attempts to define fixation speed in tissue masses is unconvincing, then there is clear evidence from the recent work of Yong-Hing, et al. and Dawe, et al. (21,22). These studies demonstrate that the histologists rule of thumb for the rate at which aldehyde fixation proceeds, which is 1 mm penetration of formalin per hour for first hour, then 1mm penetration per 3 hours for subsequent thickness at room temperature is a good approximation of the rate at which fixation proceeds in the cerebral cortex.

These studies provide robust empirical evidence for the validity of calculations of the penetration rate of methylene glycol that were carried out by Tellyesniczky (23) in 1910 and confirm the empirical results obtained in model systems such as tissue blocks and plasma blocks.

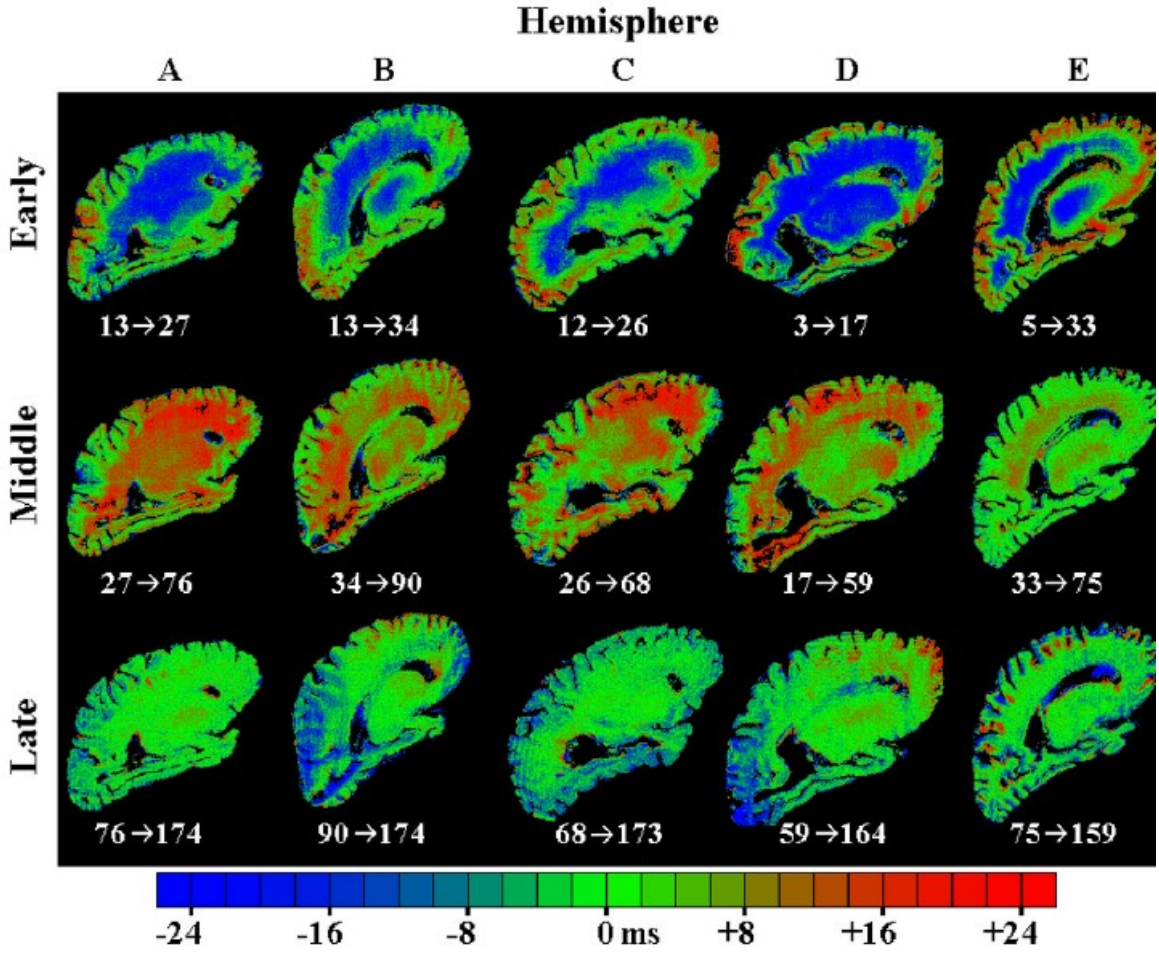


Figure 4.
T_2 difference maps for all five hemispheres, showing the changes in T_2 that occur during three different postmortem time periods. The early phase is defined as the time over which T_2 decreased in deep tissue of each hemisphere. The middle phase corresponds to the subsequent gradual rise in T_2 values of deep tissue over the next one to two months, and the late phase corresponds to the T_2 plateau that was reached two to three months postmortem. The numbers below each image indicate the time points (in days postmortem) that were compared in order to generate that image.

Above is an image from the Dawe, et al., which dramatically illustrates the slow penetration of methylene glycol into the brain during immersion-fixation as evaluated by T_2 MRI. As can be seen fixation is not complete until 2-3 months at 4°C. Three months at 4°C is presumably ample time for autolysis to occur in brain tissue stored at this temperature. One possible mitigating factor might be that the lipids in the cells will have undergone phase transition well above 4°C and thus will be in a solid or semi sold state, presumably rendering

them more resistant to enzymatic attack. For example, Ding, et al., Shatil, et al., and Liu, et al., claim good histological and substantial axonal preservation deep inside immersion fixed brains (24,25,26).

Alternatively, D'Arceuil, et.al. (27) evaluated rat brain autolysis at 4°C using MRI, which found that there are large decreases in the fractional anisotropy and the apparent diffusion coefficient during the first 24 hours of a postmortem interval of 0-14 days at 4°C as can be seen in the graphs from this below.

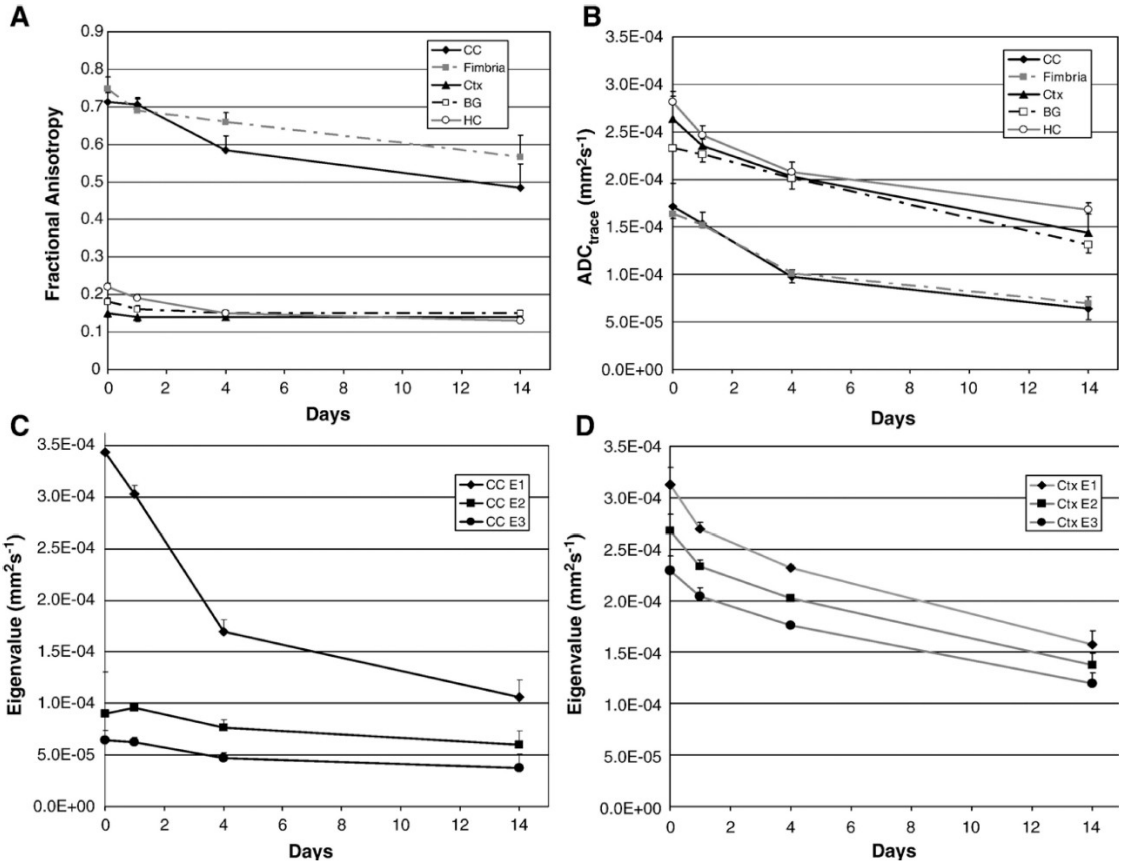
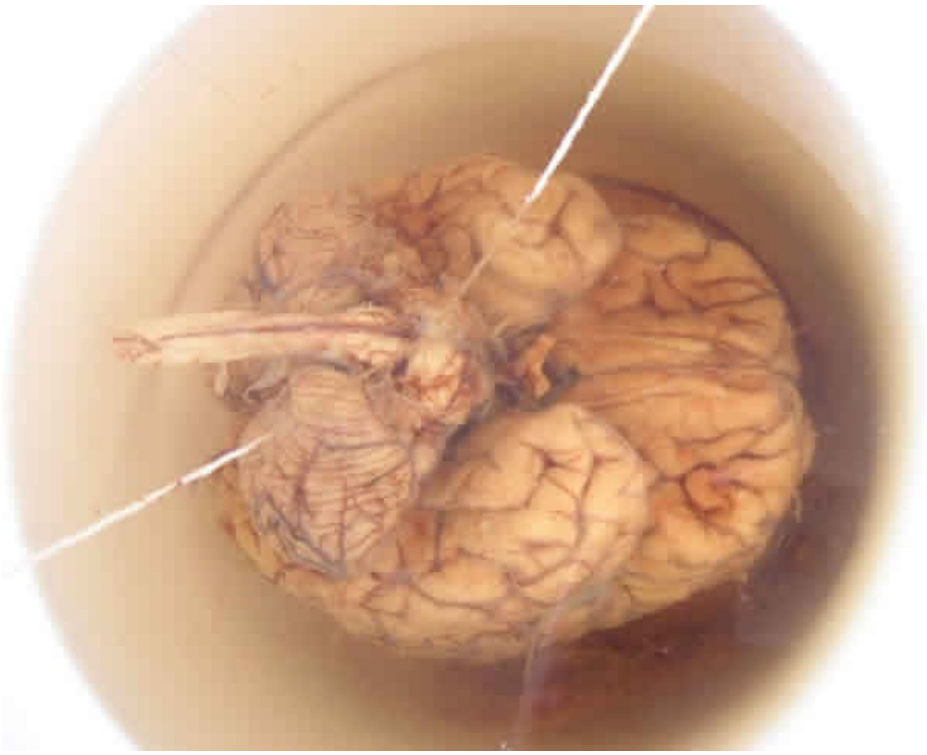


Fig. 1.
FA (A) and ADC (B) variation in gray (frontal cortex, basal ganglia and hippocampus) and white matter (corpus callosum genu and fimbria) with increasing postmortem interval (PMI). Eigenvalues (absolute value) in white (C) and gray (D) matter decreased with increasing PMI. Error bars are the standard deviation of the measurement.

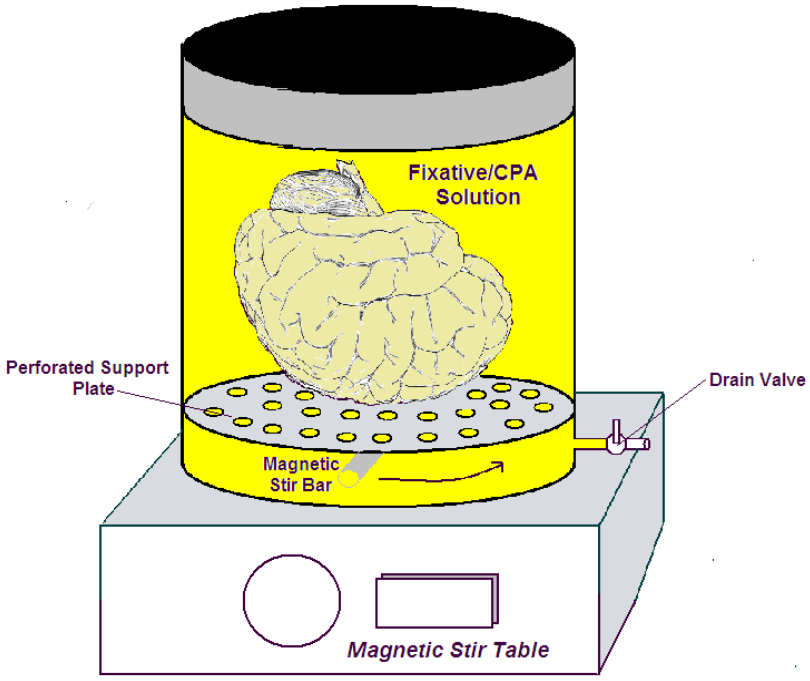
Similarly, Hukkanen, et al., found that myelin was found to have degenerated after an incubation of 24 hr at +4 and +25°C using TEM in human brain tissue samples recovered from neurosurgical procedures on living patients (28).

Also, in contrast to the earlier studies cited showing substantial biochemical stability over long PMIs, it bears emphasizing that there are many contrary studies indicating substantial degradation of brain biochemistry, including of structural proteins, over a time course of 4-24 hours at 4°C. A representative few of these studies are listed here: 29, 30, 31.

Immersion fixation of the brain as practiced by pathologists consists of suspension of the brain from a string attached to one of the vessels emanating from the circle of Willis in a pail of unstirred, buffered formalin, as illustrated below.

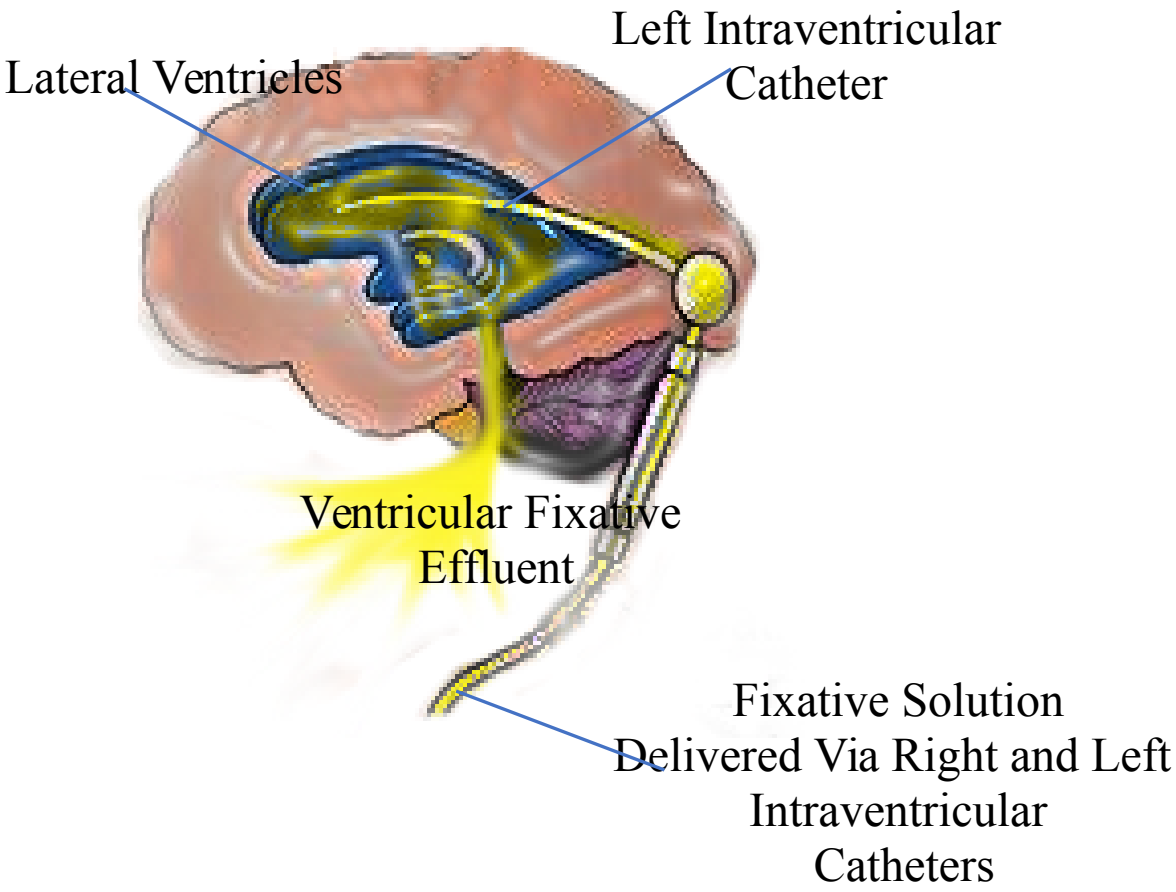


The speed with which fixation proceeds using this procedure should be increased if the fixative is stirred in an apparatus such as the one shown below.



Continuous stirring of the fixative will prevent both the formation of boundary layers around the brain, as well as any stratification that may occur as water is transiently, osmotically removed from the brain.

The time for diffusion of fixative into the deep brain might be further reduced by placing catheters into the left and right lateral cerebral ventricles to allow for continuous perfusion/irrigation of fixative solution into the interior of the brain, thus decreasing the time for the fixative chemicals to reach equilibrium with both the cerebral cortex, the white matter and the midbrain:



Right and left lateral cerebral intraventricular catheters may be placed as soon as possible after taking custody of the patient in order to facilitate more rapid and even equilibration of the brain with fixative and cryoprotectant.

With the advent of compact and relatively inexpensive portable ultrasound devices that can easily interface with a laptop computer it may be possible to safely place intraventricular irrigation catheters in the field, thus further improving the speed and quality of fixative preservation:

Compact, hand-held, battery operated ultrasound unit with integral monitor shown here connected to a laptop computer. These devices allow for excellent in-field visualization of the cisternal structure in the interior of the brain, thus facilitating safe and accurate placement of fixative irrigation cannulae in the lateral cerebral ventricles.

ARTERIAL FIXATIVE FILLING AS AN ALTERNATIVE TO IMMERSION FIXATION?

Overcoming No-Reflow

While Ames, et al., discovered the no-reflow phenomenon, it was Hossman, et. al., who discovered how to overcome it (32, 33).

They were able to demonstrate a return of cerebrocortical activity after 1-hour of normothermic ischemia in the cat. The primary mechanism they used to do this was by increasing the arterial pressure from the normal, resting MAP of~70 mmHg, to ~150 mmHg.

One of the reasons these investigators have given as the inspiration to conduct this research was because it had long been known that it was possible to obtain reasonable cerebral angiograms in brain dead patients. That's why dye contrast angiography is not used in the determination of brain death.

The reasons for this, as Flowers, et al., (34) noted in 2009 are as follows:

"All 10 reasons given for persistent blood flow after brain death have one thing in common: the intracranial pressure or pressure outside the visualized vessels does not exceed systolic pressure or the pressure inside the vessels. This cause for filling or nonfilling of intracranial arteries in brain death was not always obvious. Initially, vascular occlusion and spasm were proposed as major factors. In 1961, Pribram[29] reported 11 well-documented cases of acute intracranial hypertension. He surmised that an acute rise in intracranial pressure was responsible for non-filling of the cerebral vessels by angiography and suggested that in similar cases, lowering of the intracranial pressure would permit visualization of the cerebral vessels. In 1962, Mitchell et a[39] studied this phenomenon experimentally in monkeys and dogs. When the intracranial pressure was equal to or exceeded the systolic blood pressure, cerebral arteries did not fill. The complete circle of Willis, including the basilar artery, was shown by forceful injection, suggesting total interruption of intracranial flow, since this angiographic pattern does not occur with normal intracranial pressure. If the intracranial pressure was lowered below the systolic blood pressure, cerebral vessels filled well. Arteriograms made at an intracranial pressure below the recorded systolic blood pressure appeared no different from the control (normal) arteriograms.

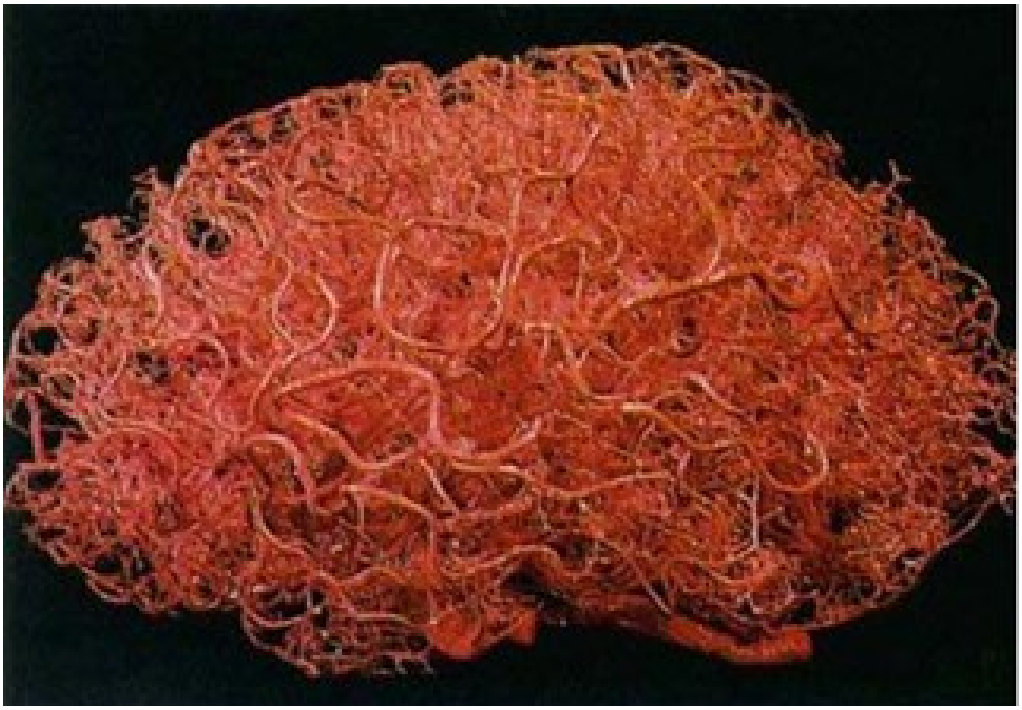
29. Pribram HW: Angiographic appearances in acute intracranial hypertension. Neurology 1961; 11:10-21

39. Mitchell OC, de la Torre E, Alexander E, et al: The nonfilling phenomenon during angiography in acute intracranial hypertension. J Neurosurg 1962; 19:766-774

This suggested that it might be possible to overcome no-reflow if the MAP was raised to a high enough level, possible in conjunction with a craniotomy, to relieve pressure and this is what Hossmann, et al., did.

But it also suggests something else, and that is that it is possible to achieve angiographic filling of the brain vasculature even after very long PMIs. After all, these subjects were all brain dead, and in most cases had been so for several, and in many cases after many hours after circulation to the brain had ceased.

There is other compelling and graphic evidence that extensive filling of the cerebral arterial vasculature is possible after long PMIs, as demonstrated by the successful corrosion casting of the human cerebral vasculature, as shown in the photo below (35).



The extraordinary things to consider about this about this cast and others like it are that:

* they were made hours after clinical death.

* there is excellent filling of the cerebral arterial vasculature, at least to the level of the millimeter, (and possibly) submillimeter arterioles.

* the casting material is far more viscous than blood (more on that shortly).

The first and potentially most important implication to consider as a result of the observations above is that the aldehyde fixatives, unlike cryoprotectants, need to be present only in comparatively miniscule concentrations. Cryoprotection for vitrification, and for most cryobiological applications, works because of the colligative action of the CPAs. In other words, they inhibit ice formation largely as a function of the degree to which they replace water in solution, plus some added benefit gained from hydrogen bonding which contributes to glass formation.

Thus, it isn't enough to simply "replace the blood" with antifreeze (i.e., fill the vessels) to achieve cryoprotection in the brain or other organs and tissues, it is necessary to replace a significantly fraction of the total water in the cells and tissues. This is not the case with fixatives since they are able to fill the arterial tree (as per above) which might result in timely enough and adequate enough fixation, providing that the terminal fixative concentration in the vasculature was high enough to allow for diffusion of fixative from the vessels sufficiently well to fix the brain parenchyma. This might be so (especially under conditions of refrigeration where autolysis has been greatly slowed) because the fixative will eventually diffuse out from the vessels into the tissue, whether the vessels are of small, medium or large caliber. Thus, in this scenario, it would be a race between the limits of the protective effects of refrigeration and the penetration of the aldehydes.

The next question is just how small a caliber of vessels is it possible to fill in this manner? There is a small and growing literature on postmortem angiography and if that literature is examined for the evidence of small vessel filling in the brain there is evidence that it is possible to achieve reasonably uniform capillary filling with *high viscosity* angiographic material perfused at supraphysiologic pressures:

As Jackowski, et al. (42) state:

"For the purpose of studying of the structure of the brain blood vessels and the pathologic changes of them simultaneously, a new method using barium sulphate has been carried out.

This method is effective not only for observation of the extracranial large vessels but also the capillaries of the brain. Relation between quantity of barium sulphate and radiological finding of brain capillaries depends on the pressure & speed of injection and the vessel to be injected dye."

Similarly, as Ascwin deWolf has observed in his ischemic rats, the higher the viscosity of the perfusate (or contrast agent, in this case), the better the filling/perfusion of ischemic vessels. In 2006, Grabherr, et al., took this observation to the extreme and dissolved their contrast media in diesel fuel, which they subsequently perfused cadavers with (43).

Since then, there have been other successful efforts to facilitate much improved postmortem perfusion by deliberately increasing the viscosity of the contrast media/perfusate with ethylene and by the use of polyethylene glycol (44).

The quote below is from Preusser, et al. (45):

“Disclosure of neurological disorders by neuropathological examination may be one important aim of forensic autopsies. There are insufficient data on human brain tissue preservation after prolonged postmortem periods. Here, we describe neuropathological findings in a brain of a 77-year-old woman that was fixed at autopsy 2 months after death. The body had been stored in a cooling chamber at 3°C temperature. Gross inspection of the brain was satisfactorily possible.

Histomorphology was excellently preserved. Many histochemical and immunohistochemical stains allowed satisfactory neuropathological evaluation of brain tissue and the diagnosis of Alzheimer's disease. Nevertheless, some immunohistochemical stainings repeatedly yielded negative or suboptimal results.

We conclude that neuropathological examination of human brain tissue extracted from the skull and fixed after prolonged body storage in a cool environment is feasible for forensic diagnosis of neurological disorders even several months after death. However, in such cases the significance of negative immunohistochemical staining results must be interpreted with caution.

For the purpose of studying of the structure of the brain blood vessels and the pathologic changes of them simultaneously, a new method using barium sulphate has been carried out.

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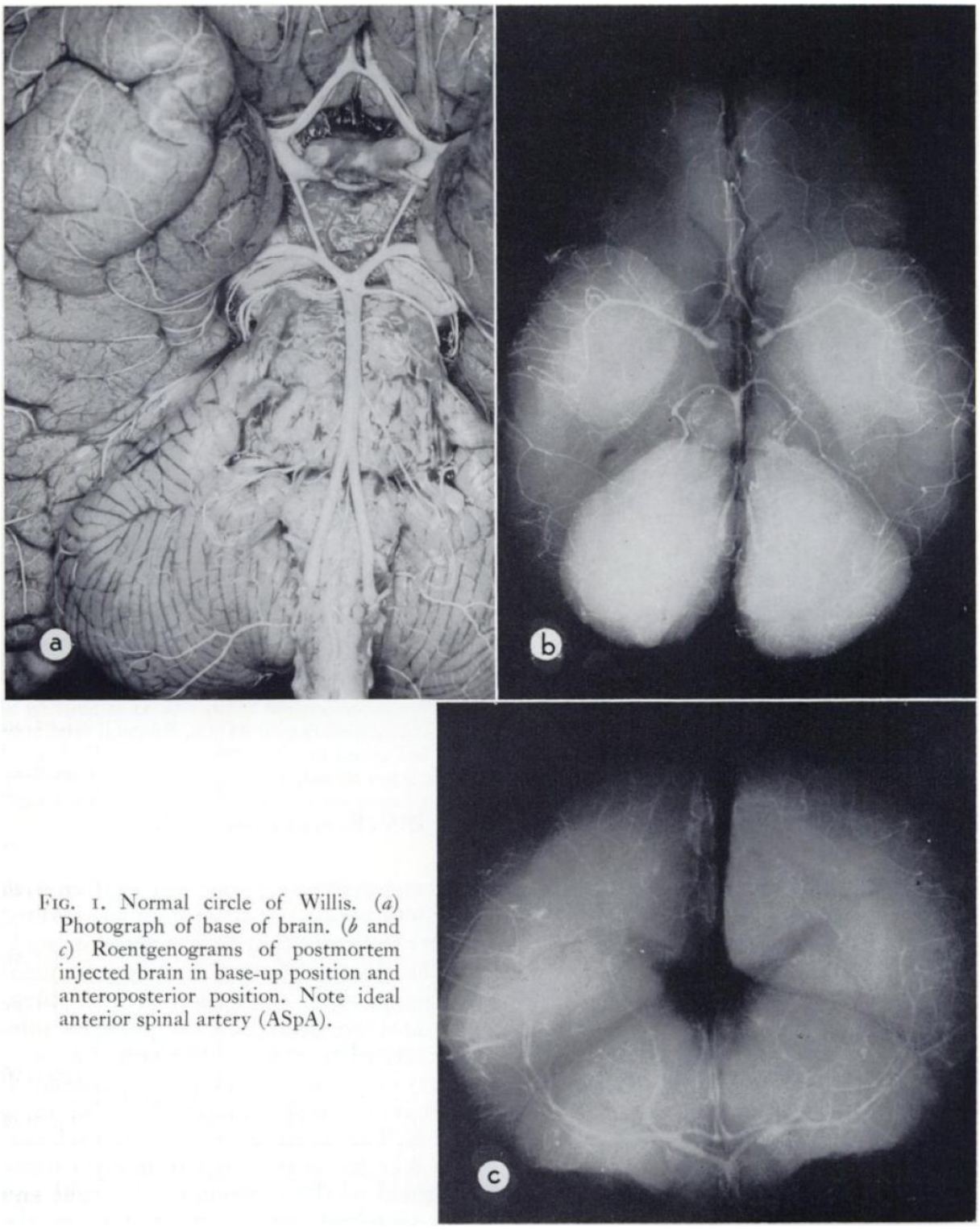


FIG. 1. Normal circle of Willis. (*a*) Photograph of base of brain. (*b* and *c*) Roentgenograms of postmortem injected brain in base-up position and anteroposterior position. Note ideal anterior spinal artery (ASpA).

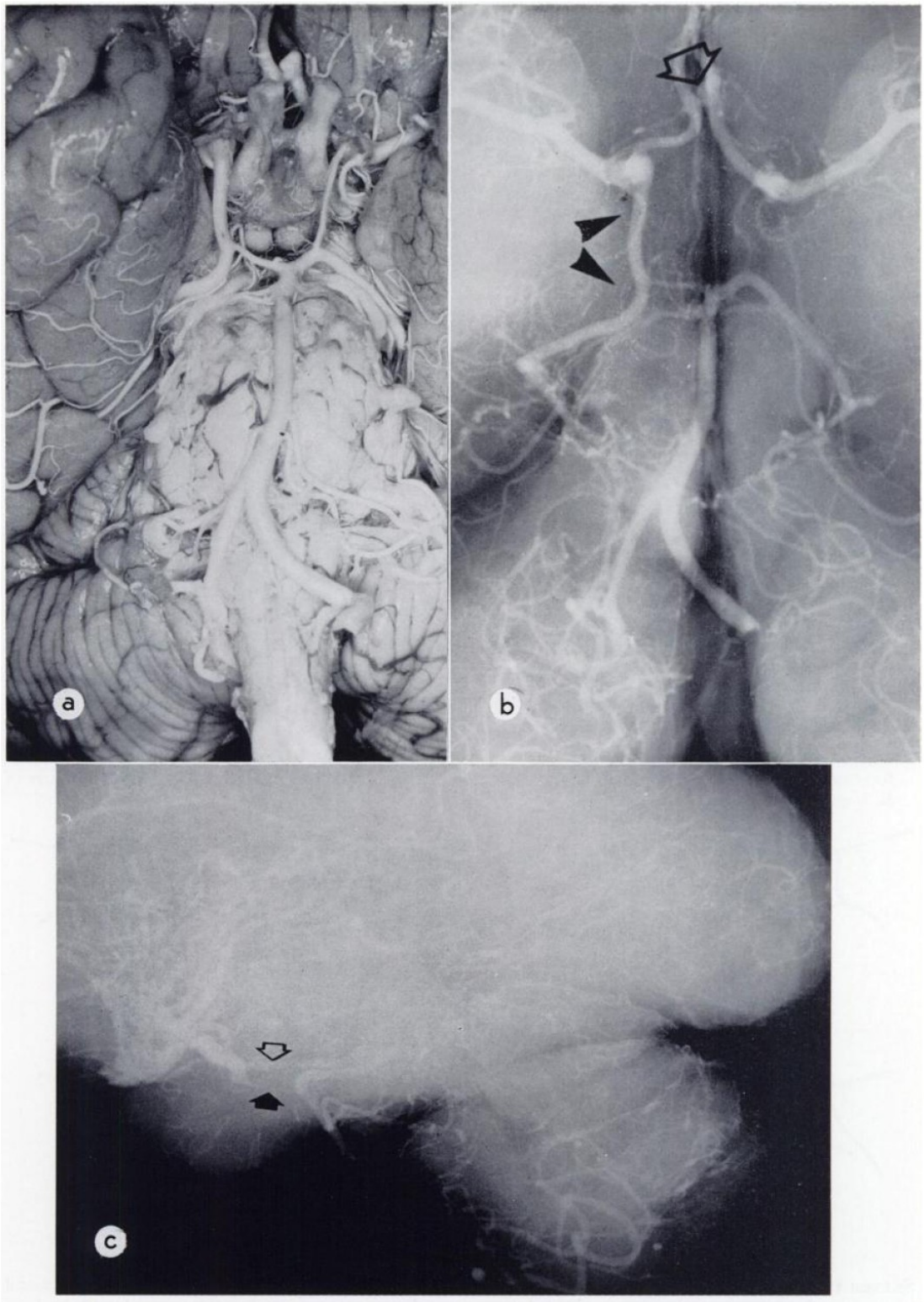
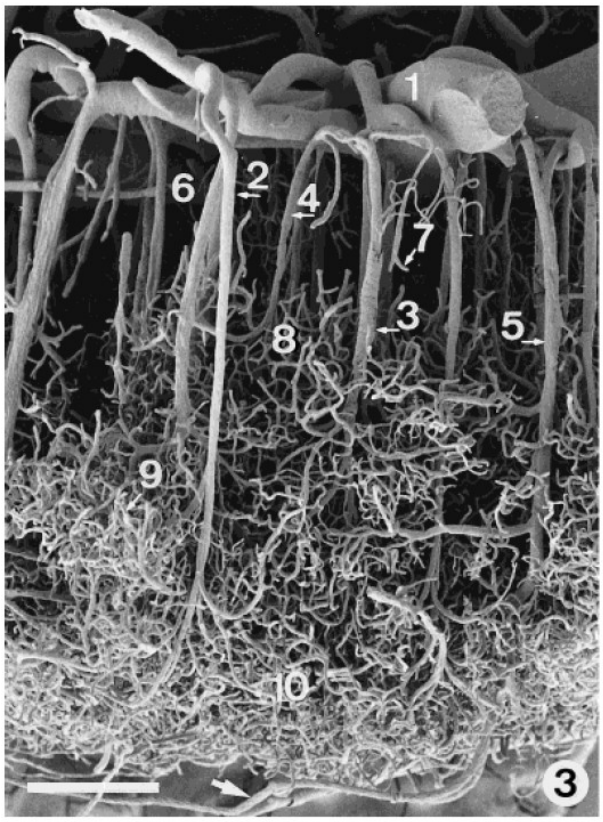


FIG. 3. (*a*) Photograph of base of brain. (*b*) Roentgenogram in base-up, and (*c*) in lateral position of brain specimen. Complete right fetal type PCA, left normal PCA. Aneurysm of ACCA. Hyperplastic left ACHA. Absent posterior inferior cerebellar artery (PIClA), left.

The images above are from Reina-De La Torre, et al. (46).

Further, Reina-De La Torre, et al. (46), has published some remarkable work where they examined the effectiveness of the filling of very small caliber cerebral vessels with corrosion casting media after PMIs that did not exceed 12 hours. The resulting SEMs are astonishing, showing excellent and uniform filling of the cerebral vessels down to the level of the arterioles: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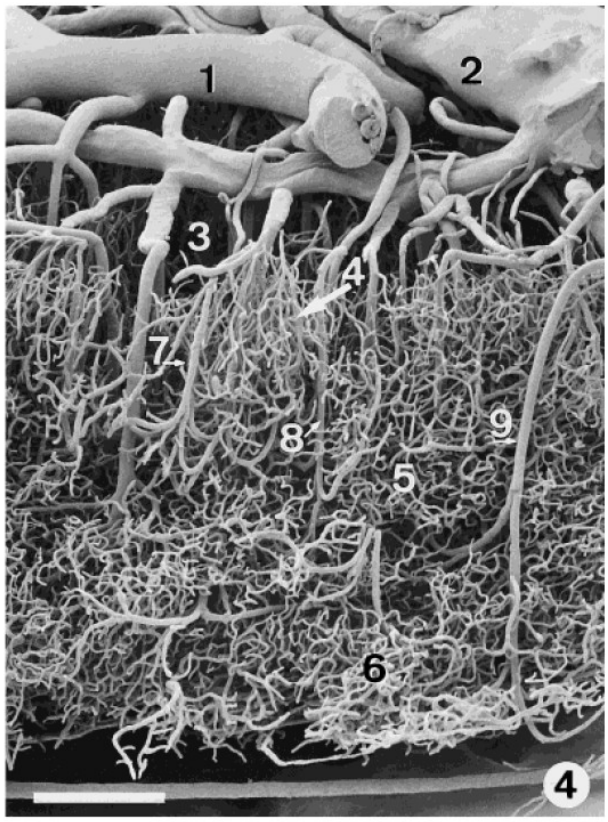
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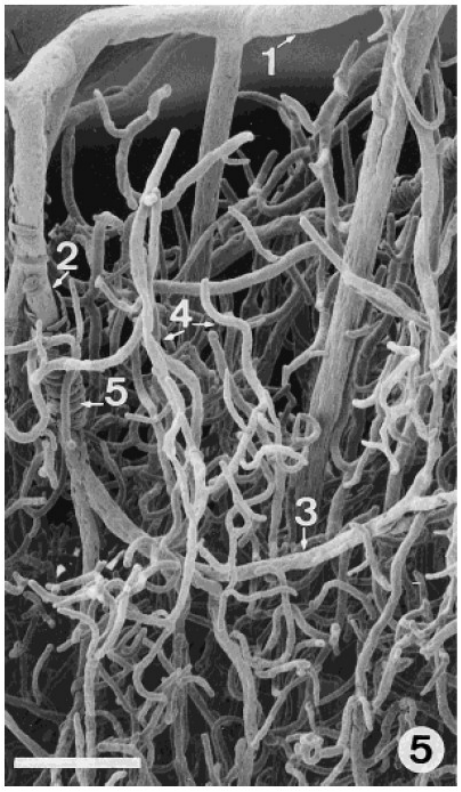
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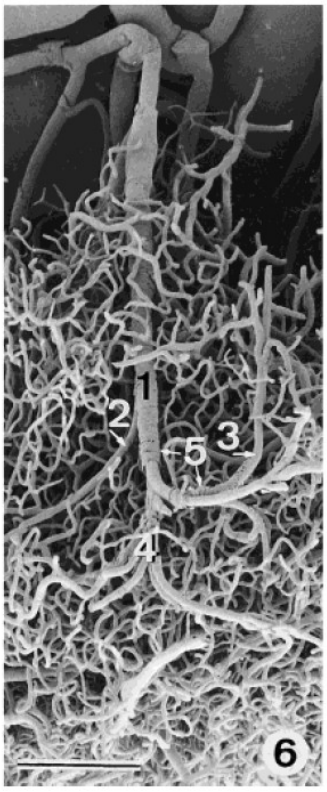
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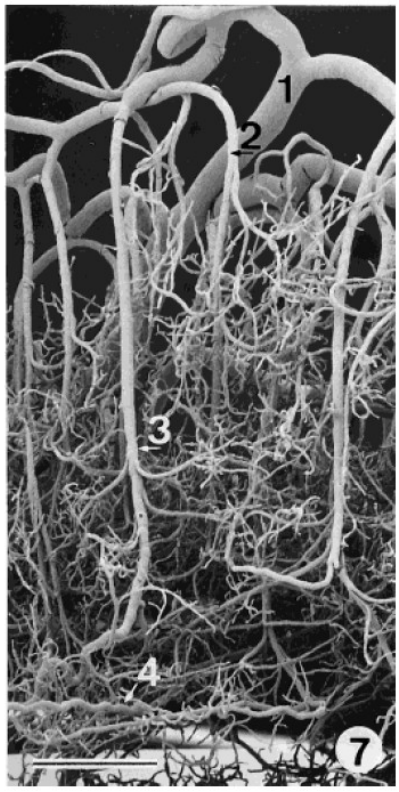
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These images, if consistently reproducible, indicate that the maximum distance in the tissue between arterial vessels should not exceed 1-3 mm. That would shorten fixation times to those in the range achieved by immersion fixation for the cerebral cortex, e.g., ~24 hours.

DISCUSSION

Immediate initiation of chemical fixation in cases where perfusion is not possible, or where the brain has already been subjected to fixative perfusion, is attractive in that it allows for subsequent introduction of cryoprotectant to a vitrifiable concentration by soaking the organ in *stirred fixative media* containing increasing concentrations of cryoprotectants (typically glycerol) over a period of months, thus eliminating any damage as a result of freezing during subsequent deep cooling. At least three human brains (cryonics patients) have been successfully vitrified in this fashion (47)) although their histological and ultrastructural condition was not examined.

One problem with this approach is determining under what conditions it is contraindicated. Evaluation of long PMIs in sheep using CT and MRI evaluation have shown that decomposition of the brain is indicated by gas bubble formation in the brain parenchyma and visible changes in image character with both T1 and T2 weighting in MRI (48). The author has observed these changes in the brains of cryonics patients where there has been a long PMI followed by immersion fixation. The presence of such findings may therefore be taken as a relative contraindication to the use of immersion fixation in such since further, rapid decomposition is typically not halted by refrigeration under these conditions. There is also evidence that the degree of autolysis at a given PMI in the human brain is subject to substantial interindividual variability. This variation i9n the degree of preservation (or autolysis) is thought to be a result of the perimortem condition of the patient as well as the cause of death (49).

Given the paucity of experimental or clinical data available at this time much additional animal and cadaveric research is imperative. In particular, it will be important to determine the effectiveness of arterial (and possibly venous) vascular filling with fixative, and the extent to which interindividual differences and other factors affect the quality and consistency of the outcome.

Currently in cryonics cases where it is possible to perfuse the brain with fixative before autopsy it is the practice to achieve post-fixation by immersion of the brain in modified Karnofsky's fixative(50) immediately after the brain is removed, followed by slow, step-wise loading with glycerol in Karnofsky's to a terminal concentration of ~75% v/v glycerol carried out at 2-4ºC.

Hypertensive hydrostatic filling of the cerebral arterial vasculature, possibly followed by low pressure hydrostatic filing of the cerebral venous vasculature, would seem an attractive alternative to immersion fixation where it is deemed impossible to achieve either cryoprotective perfusion, or adequate fixation using conventional perfusion fixation. Very generally, such a protocol would consist of static filling of the cerebral arterial (and possibly venous vasculature) followed by immersion in fixative solution containing progressively higher concentrations of glycerol while being refrigerated to 2-4ºC.

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