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Area changes in slices of rat brain during preparation for histology or electron microscopy

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SUMMARY

Cerebral slices cut from rat brain, either 2–3 mm or 0·27 mm thick, were used to study the effect of embedding and freezing. Paraffin wax sections 6 μm thick were mounted and stained with haematoxylin and eosin or Marsland *et al.*'s (1954) silver stain, and their areas were examined at each step.

Embedding in paraffin wax of slices 2–3 mm thick, or in Epon of slices 0·27 mm thick, caused a diminution of their areas by 20–30%. Staining of paraffin wax sections did not alter their areas. Glycerol alone at 15% concentration had no effect on the areas, but at 30% concentration they were diminished by approximately 20%. Diminution of the areas of glycerol treated slices 0·27 mm thick also occurred when they were transferred to liquid N_2 or to isopentane, but the areas increased after glycerol was replaced by Freon 12.

It was concluded that embedding or freezing cerebral slices caused changes in their areas, but that staining of sections after they had been embedded, sectioned and mounted did not.

INTRODUCTION

Although there is an extensive literature on histological and electron microscopic techniques, there have been relatively few studies on the effects of the reagents used in preparation on the apparent dimensions of the structures examined (Hertwig, 1931; Ross, 1953; Frontera, 1959; Kushida, 1962; Baker, 1970; Boyde *et al.*, 1977). Obviously measurements intended to assess the total or relative dimensions in living cells must take into account any changes there might be in the dimensions as a result of the reagents used. Previously, the apparent areas of the cytoplasm, the nuclei and the nucleoli have been examined at each stage after initially unfixed isolated rabbit neurons were subjected to a routine haematoxylin and eosin procedure (Hillman *et al.*, 1976). The areas of cerebral slices and unfixed isolated neurons have been observed before and after the addition of six different widely used fixatives (Deutsch & Hillman, 1977). In the present paper these observations have been extended by examining the effects on rat cerebral tissue of each stage of the following procedures: embedding in paraffin wax; embedding in 'Epon' resin; the haematoxylin and eosin procedure; a silver stain (Marsland *et al.*, 1954); the cooling of unfixed tissue with and without previous immersion in glycerol.

The particular aim of these experiments was to assess each step of typical, widely

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used histological procedures, in order to attempt to modify them in the future, to circumvent or avoid specifically those steps which most alter the tissue during preparation.

METHODS

Preparation and measurement of the areas of tissue sections

Albino rats weighing 0·2–0·3 kg were killed by placing them in an atmosphere of excess ether; their cerebral hemispheres were removed as soon as their hearts had stopped beating. The hemispheres were placed immediately in Petri dishes saturated with water vapour. Either sagittal slices 2–3 mm thick weighing 150–200 mg were cut in dry conditions using a scalpel, or thinner tangential slices weighing 20–80 mg were cut with guides with a clearance of 0·27 mm (McIlwain, 1975).

The 2–3 mm pieces of tissue were irregular, and so it was not possible to compare the areas of 6 μm sections with those of the blocks from which the sections were cut. Sections 6 μm thick were cut from tissues embedded in paraffin wax and mounted on microscope slides to which they were made to adhere; this was in contrast to all the other observations in which the 2–3 mm and 0·27 mm slices were prevented from adhering to the glass. The areas of the 6 μm mounted sections were then taken as 100% and the effects on their areas during staining with haematoxylin and eosin, or the silver stain (Marsland *et al.*, 1954), were measured as a percentage of the areas of the mounted sections.

Slices 2–3 mm thick were cut and stored in humidified Petri dishes at the following temperatures (K): 295, 277, 248, 217 (in a mixture of acetone and solid CO_2). Other slices (0·27 mm thick) were placed for the times indicated on Tables 7–9 in the following liquids: glycerol (15%); glycerol (30%); liquid N_2; glycerol (15% or 30%) replaced by liquid N_2; isopentane or Freon 12 replaced by liquid N_2; glycerol (15% or 30%) replaced by isopentane or Freon 12 and then liquid N_2. Slices 2–3 mm were also placed in liquid N_2 alone. In all these conditions, the areas of the same sections were measured before and after immersion for the times and temperatures (indicated in Tables 7–9).

In the present paper, photocopying was used to measure the area of tissue. The errors of the latter technique could arise if the reagents altered the shape of the tissue or caused its edges to curl. This possibility was minimized by floating cover slips lightly on the tissue, which, however, was prevented from adhering either to them or to the Petri dishes, except when mounted sections were being studied.

The slices were placed flat in Petri dishes by the use of a paint brush slightly moistened with liquid paraffin, which had also been used to wipe the cutting blades, the guides and the Petri dishes beforehand. Each slice was checked to ensure that it did not adhere to the Petri dish or was folded or stretched; irregular slices were trimmed to regular shapes. The Petri dishes were closed with moistened filter paper adherent to the inside of the tops to keep the atmosphere humid. The whole Petri dishes were then placed on a Rank Xerox photocopier, and photographed. The contours of the tissue sections were cut out and the pieces of paper were weighed. The initial area of each slice was taken as 100%, and at each stage of preparation, the areas were expressed as a percentage of this original area. Some 2–3 mm and 0·27 mm slices were each photocopied eight times, their areas were calculated, and the errors of the measurements of individual slices are given in Table 1.

The steps of the procedures used are given in each table. The number of slices vary as some were lost, damaged or folded by the addition of particular reagents. All figures given are the mean and standard deviation of the number of slices or sections indicated in brackets in the Tables, and the significance was assessed by the Student '*t*' test.

Table 1. Error of measurement of photostat copies of the projected areas of slices. Each copy of an individual specimen was made eight times.

Slices 2–3 mm thick		Slices 0·27 mm thick	
Mean areas of slices (mm^2)	Error (%)	Mean areas of slices (mm^2)	Error (%)
38	±3	30	±3
41	±5	42	±4
90	±2	80	±2
76	±2	92	±2

In Tables 2–5 the *same* tissue was used throughout the procedure. In Tables 2–9 all *P* values are the significances of the changes relative to the areas of the fresh unfixed tissues in all Tables except 4 and 5, where it is relative to the sectioned and mounted tissue. Dashes in Tables indicate that the measurement could not be made.

Individual techniques

The 2–3 mm slices were embedded in wax, but only the 0·27 mm slices were embedded in Epon, as it is essential in electron microscopy—in which Epon is used—that the reagents can diffuse into the tissue rapidly.

RESULTS

The results are summarized in Tables 2–9.

Table 2. The effect of embedding the tissue in paraffin wax. The areas of forty-three slices 2–3 mm thick were examined during the stages of embedding.

Reagent used and its concentration	Duration of immersion (h)	Areas of slices as % of initial areas (mean ± SD)	*P* value of change
(Unfixed)		100	
Buffered formalin (10%)	3·5	132 ± 25	< 0·001
Ethanol (50, 70, 90, 100%)	1·0	86 ± 28	< 0·01
Xylene (100%)	0·5	84 ± 18	< 0·001
Paraffin wax	24·0	67 ± 16	< 0·001

Table 3. The effect of embedding in Epon on the areas of eighteen unfixed slices 0·27 mm thick.

Reagent and its concentration	Duration of immersion (min)	Areas of sections as % of initial areas (mean ± SD)	*P* value of change
(Unfixed)		100	
Glutaraldehyde (4%)	180	90 ± 10	n.s.
Rinsed with cacodylate (0.1 M)	0·1	—	
OsO_4 (1% w/v)	60	92 ± 10	n.s.
Rinsed with cacodylate (0.1 M)	0·1	—	
Ethanol (50, 70, 90, 100%)	40	69 ± 15	< 0·001
Propylene oxide	30	—	
Propylene oxide (50%):Epon (50%)	30	71 ± 15	< 0·001
Epon (295 K)	20	79 ± 11	< 0·001
Epon (333 K)	48 h	76 ± 16	< 0·001

DISCUSSION

Errors of measurement

The area of the tissue was measured only in plan view, because this is the normal orientation in which it is examined throughout histology, histochemistry and electron microscopy. Since the tissue is organized in layers so that its structure appears different in longitudinal and transverse section, the present study does not give information about changes in projected area in planes other than those studied.

Table 4. The effect of dewaxing, rehydrating, staining and embedding on the areas of seventeen paraffin wax sections cut 6 μm thick during the haematoxylin and eosin procedure. In this Table and Table 5, sections were mounted on, and adherent to, the slides throughout the observations, and their areas sectioned and mounted before staining were taken as 100%.

Condition	Duration of condition (min)	Areas of sections as % of sectioned and mounted areas (mean ± SD)	*P* value of change
Paraffin wax sections		100	
Dewaxed with xylene (100%)	10	112 ± 9	n.s.
Rehydrated with ethanol (100, 90, 70, 50%) then water	5	—	
Stained with haematoxylin (1%)	4	103 ± 9	n.s.
Rinsed in distilled water	0·1	—	
Rinsed in acid alcohol	0·1	—	
Rinsed in tap water	5	—	
Stained in eosin (1%)	4	103 ± 1	n.s.
Ethanol (70, 90, 100%)	5	108 ± 5	n.s.
Xylene (100%)	20	108 ± 6	n.s.
Embedded in DPX	18 h	107 ± 7	n.s.

Table 5. The effect of dewaxing, rehydrating, staining and embedding on the areas of ten sections, cut 6 μm thick, during staining by the method of Marsland *et al.* (1954).

Condition	Duration of condition (min)	Areas of sections as % of sectioned and mounted areas (mean ± SD)	*P* value of change
Paraffin wax sections		100	
Dewaxed with xylene (100%)	10	108 ± 8	n.s.
Rehydrated with ethanol (100, 90, 70, 50% then water)	5	—	—
Silver nitrate (12%)	1	101 ± 7	n.s.
Formalin (10%)	1	99 ± 4	n.s.
Silver nitrate (12%)	0·5	95 ± 7	n.s.
Formalin (10%)	1	92 ± 8	n.s.
Rinsed in distilled water	0·1	—	
Thiosulphate (5%)	1	87 ± 5	< 0.02
Ethanol (50, 70, 90, 100%)	5	98 ± 8	n.s.
Xylene (100%)	10	98 ± 7	n.s.
DPX	18 h	93 ± 12	n.s.

Table 6. The effect of different temperatures in a humid atmosphere on unfixed slices 2–3 mm thick. The numbers of samples are indicated in parentheses.

Temperature (K)	Time at that temperature (min)	Areas of slices as % of initial areas (mean ± SD)	*P* value of change
295	45	106 ± 17 (25)	n.s.
277	45	108 ± 11 (18)	n.s.
248	45	118 ± 10 (18)	< 0·01
217	5	74 ± 14 (22)	< 0·001

Table 7. The effect of immersion in glycerol on the areas of unfixed slices 0·27 mm thick. Twelve sections were followed in each concentration of glycerol. In Tables 7–9 the tissue was immersed in glycerol which was initially at room temperature (293–295 K).

Glycerol (15%)			Glycerol (30%)		
Duration of immersion (min)	Areas of slices as % of initial areas (mean ± SD)	*P* value of change	Duration of immersion (min)	Areas of slices as % of initial areas (mean ± SD)	*P* value of change
20	95 ± 10	n.s.	20	80 ± 5	< 0·001
30	100 ± 7	n.s.	30	81 ± 7	< 0·001
60	97 ± 12	n.s.	60	83 ± 7	< 0·001
90	93 ± 12	n.s.	90	79 ± 9	< 0·001
120	98 ± 13	n.s.	120	85 ± 11	< 0·001

Table 8. Effect of liquid nitrogen in the absence and presence of glycerol. Slices were photographed, then immersed in liquid N_2 and photographed again. This procedure was repeated after immersion in glycerol (15%) or glycerol (30%) which was drawn off before addition of liquid N_2. The slices did not adhere to the Petri dish. The number of slices is given in parentheses.

Slice thickness (mm)	Condition	Duration of condition (min)	Areas of slices as % of initial areas (mean ± SD)	*P* value of change
2–3	Liquid N_2 (77 K)	5	59 ± 15 (8)	< 0.001
0·27	Liquid N_2 (77 K)	5	104 ± 15 (34)	n.s.
0·27	Glycerol 15% (295 K)	20	95 ± 10 (10)	n.s.
	Replaced by liquid N_2 (77 K)	5	61 ± 7	< 0·001
0·27	Glycerol 30% (295 K)	20	80 ± 5 (10)	< 0·001
	Replaced by liquid N_2 (77 K)	5	47 ± 8	< 0·001

Table 9. The effect of glycerol, cooled isopentane, Freon 12 and liquid nitrogen. Slices 0·27 mm thick were photographed, then immersed in isopentane or Freon cooled to 113 K with liquid N_2; the isopentane or Freon was removed and replaced by liquid N_2. This procedure was repeated after immersion in glycerol (15%) or glycerol (30%) which was drawn off before addition of isopentane, which itself was drawn off before addition of isopentane, which itself was drawn off before addition of liquid N_2. The numbers of slices are given in parentheses.

Condition	Duration of condition (min)	Areas of slices as % of initial areas (mean ± SD)	*P* value of change
Isopentane (113 K)	5	89 ± 11 (11)	n.s.
Replaced by liquid N_2 (77 K)	5	82 ± 18	n.s.
Glycerol 15%	20	104 ± 12 (9)	n.s.
Replaced by isopentane (113 K)	5	87 ± 13	0·05
Replaced by liquid N_2 (77 K)	5	80 ± 16	0·05
Glycerol 30%	20	77 ± 8 (10)	0·001
Replaced by isopentane (113 K)	5	82 ± 3	0·001
Replaced by liquid N_2 (77 K)	5	72 ± 2	0·001
Freon 12 (118 K)	5	101 ± 6 (16)	n.s.
Replaced by liquid N_2 (77 K)	5	87 ± 7	0·001
Glycerol 15%	20	102 ± 7 (9)	n.s.
Replaced by Freon 12 (118 K)	5	193 ± 17	< 0·001
Replaced by liquid N_2 (77 K)	5	166 ± 12	< 0·001
Glycerol 30%	20	81 ± 5 (9)	< 0·001
Replaced by Freon 12 (118 K)	5	111 ± 8	< 0·01
Replaced by liquid N_2 (77 K)	5	93 ± 6	n.s.

A brief discussion of other errors in histology was given in a previous paper (Deutsch & Hillman, 1977).

Embedding

Embedding in paraffin wax resulted in a diminution of area of the slices 2–3 mm thick as used in histology by a mean of 33%, and in Epon of slices 0·27 mm thick as prepared for electron microscopy by a mean of 24% (Tables 2 and 3).

During embedding, the nuclei of amphibian erythrocytes and rabbit neurons have been shown to change differently from the cytoplasm (Tooze, 1964; Hillman *et al.*, 1976) and in some fixatives whole cerebral slices diminish in area differently from the nuclei or cytoplasm of neurons (Deutsch & Hillman, 1977). If this phenomenon of differential volume changes of subcellular components should be found to be general, all measurements of dimensions made in embedded tissue would have to be corrected accordingly.

Different tissues from various species do not shrink to the same extent with the same *embedding* media—a few examples are given in Table 10. Other authors have

Table 10. Values from the literature of the linear dimensions of different tissues after embedding in paraffin wax, or epoxy resins, compared with the fresh tissue. When the values were not expressed linearly, they have been calculated by taking the square roots of the areas† or the cube roots of the volumes*.

Preparation	Species	Embedding medium	Linear dimension after embedding (%)	Reference
Whole brain	Macaque monkey	Paraffin wax	88*	Frontera (1959)
Cerebral slices (20–30 mm thick)	Rat	Paraffin wax	82†	Present paper
Isolated neurons	Rabbit	Paraffin wax	50†	Hillman *et al.* (1976)
Renal cortex slices	Rabbit	Paraffin wax	87*	Stowell (1941)
Spermatocytes	*Helix aspersa*	Paraffin wax	67	Ross (1953)
Lymphocytes	Human	Epoxy resin	89	Schneider (1976)
Cerebral slices	Rat	Epoxy resin	91†	Present paper
Erythrocytes	Newt	Epoxy resin	83–111†	Tooze (1964)
Egg	Sea urchin	Epoxy resin	90–95	Kushida (1962)

examined volume, area and weight changes, under a variety of different complete histological procedures, and have found a wide range of dimensional changes (for example, Hertwig, 1931; Stowell, 1941; Lodin *et al.*, 1967; Baker, 1970; Eins & Williams, 1976).

Staining

The tissues which had been embedded, sectioned and mounted on slides did not change significantly during subsequent staining with haematoxylin and eosin, or with the silver stain (Tables 4 and 5), whereas the projected areas of cell bodies of isolated neurons did continue to diminish with the former stain (Hillman *et al.*, 1976). The neuron cell bodies were not embedded until they had been stained. The difference between the two preparations was that in the paper of Hillman *et al.* (1976) the cell bodies were not embedded initially, since they were not going to be sectioned; thus they could contract freely during staining, in contrast to the present paper in which the embedded tissue was cut and the sections made to adhere to glass slides before staining; this would prevent the whole section contracting to a greater extent than had already occurred during the embedding. Thus, one is led to conclude that embedding is the main step in a routine histological procedure which causes contraction of tissue.

Cooling and glycerol treatment

Specimens kept in a humid atmosphere for 25 min at 295 K did not undergo changes in area but there was a slight increase in area at 248 K, and at 217 K there was a mean contraction of 25% of the initial area after 5 min. A temperature of 248 K is often used in cryostat sectioning. Evidently, the effect of cold here is non-linear (Table 6). Glycerol is used as a cryoprotectant; at a concentration of 15% it did not alter the area of slices 0·27 mm thick at room temperature for up to 120 min, but a 30% solution caused a diminution of area of about 20% which did not change with time (Table 7). However, the same tissue which had been immersed in either concentration of glycerol and then in liquid N_2 showed considerable and highly significant diminution of area, although these slices 0·27 mm thick did con-

tract if immersed directly in liquid N_2; this is in contrast to the slices 2–3 mm thick, whose areas diminished considerably after direct immersion in liquid N_2 (Table 8). Thus the degree of contraction depended on the slice thickness.

Often tissue is immersed in glycerol as a cryoprotectant and then transferred to isopentane or Freon cooled in liquid N_2. The areas of cerebral slices in 15% glycerol diminished to a mean of 87% on replacement by isopentane, and subsequently to a mean of 80% when immersed in liquid N_2 (Table 9). Although, as in Table 8, 30% glycerol diminished the areas of slices, subsequently replacement by isopentane and by liquid N_2 did not produce a significantly greater degree of contraction relative to the tissue in glycerol (Table 9). Slices in cooled isopentane without cryoprotectant contracted slightly but significantly and subsequent immersion in liquid N_2 caused a slight further diminution of area, which was then significant relative to the fresh tissue ($P=0{\cdot}05$). Thus, the presence of isopentane did seem to prevent some of the diminution of area of slices 0·27 mm thick, when immersed in liquid N_2 (cf. Tables 8 and 9).

Freon cooled to 118 K did not itself change the area of the tissue, though subsequent treatment with liquid N_2 diminished the area; however, if previously treated with 15% or 30% glycerol, the area *expanded* considerably ($P<0{\cdot}001$), and subsequently diminished after addition of liquid N_2. Nevertheless, with 15% glycerol, there was still a highly significant expansion of a mean of 66% after the latter treatment compared with the fresh area ($P<0{\cdot}001$). Thus, 15% glycerol is not suitable for cerebral tissue to prevent the changes in dimensions which would occur in frozen preparations for electron microscopy (Table 9).

Boyde *et al.* (1977), using critical point drying, showed that the whole limbs and brains from embryo pigs contracted with ethanol–CO_2, acetone–CO_2, Freon 13, and Freon 13–CO_2, but their results cannot be precisely compared due to the differences in composition of tissue, age and species of animal between their experiments and the experiments presented in this paper.

Formalin fixation caused increase in tissue area of thick slices before diminution during dehydration (Table 2) and 30% glycerol plus Freon 12 also caused an expansion before the liquid N_2 diminished the areas of the slices, so that they were not finally significantly different from their initial areas (Table 9). Such alterations of area may cause distortions in structure and redistribution of diffusible ions and molecules, even when the whole procedure results in no net change.

Tooze (1964) studied the areas of amphibian erythrocytes using a Ca^{++} free- and a Ca^{++}-containing osmium fixative. Whether or not the area of the whole cell or the nucleus increased or decreased initially depended upon the species of amphibian, the pH of the fixative and the presence of Ca^{++}, but he—like all authors—found that the final dehydration always caused a shrinkage of the cells, though not always back to their initial areas.

The present study has dealt only with the changes in area of tissue, but histological and electron microscopic preparation also causes considerable biochemical changes in tissue (see, for example, Gersh, 1959; Tooze, 1964; Hopwood, 1969; Baker, 1970). Obviously, shrinkage in cells whose compartments contain different concentrations of water and other major constituents, must result also in changes of morphology of those cells. Therefore any studies of morphology, or any measurements of dimensions, in histological, histochemical or electron microscopical preparations, should be controlled by examination of the effects of the reagents used during each step of specimen preparation. Such changes as are detected in the control experiments made should be used in the assessment of the morphology and dimensions of the components of unfixed tissue.

It should be stressed that these findings would not be relevant to histopathological

studies in which healthy tissues are subjected to the same preparative procedures as are the pathological specimens.

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ADDENDUM

The outlines of the photostat images were sharp, and were not affected by the quantity of carbon within these outlines. Such variation as there was of the areas would be included in the error of the measurement eight times of the same slices (Table 1).

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