THE QUANTITATIVE HISTOCHEMISTRY OF THE BRAIN

HISTOLOGICAL SAMPLING*

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The structural complexity of the brain makes the interpretation of gross biochemical data seem almost hopeless. Obviously the situation demands study by the various histochemical methodologies available.

The general approach of Linderstrøm-Lang and Holter *et al* appeared to offer several advantages for this purpose. The original procedure was as follows: Frozen sections were cut of constant thickness. Alternate sections were analyzed by a microchemical procedure for the desired constituent, and the intervening sections were fixed, stained, and the percentage of different cell types determined by random cell counts. Using this indirect histological control it was possible to determine which cell types contain the constituent concerned. Thus pepsin, for example, was conclusively assigned to the chief cells of the stomach and the average amount per cell was calculated in various functional states (4).

Because of the great complexity of the brain, and the changes in structure which occur within short distances, it was felt desirable to establish direct rather than indirect histological control.

This has been accomplished by certain changes in technique, with a considerable increase in convenience. The final procedure is an elaboration of one developed for a study of the retina (1).

The revised technique is described, together with means for measuring the volume and dry weight of fragments of sections to be analyzed.

With the brain it is usually difficult to isolate regions larger than a few micrograms in weight which are sufficiently simple in structure to repay analysis for histochemical purposes. This necessitates the use of rather small scale chemical methods. A number of these are described elsewhere (10, 11, 12).

EXPERIMENTAL

1. *Preparation of tissue blocks.* Tissue is rapidly removed from the animal. Pieces, preferably not more than a few millimeters in least dimension are placed on strips of hardened filter paper for identification and are plunged into liquid nitrogen or a large volume of isopentane chilled to a slush with liquid nitrogen (3). The frozen tissue is allowed to warm up in small Petri dishes to the temperature of a cold room maintained at -10 to -20°C. (From the time of freezing until the sections are dry, the temperature of the tissue can never be allowed to rise above -10°C.)

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The desired blocks are cut out with a thin razor blade and are mounted as follows: A paste, made by mashing surplus brain tissue, is applied generously to the end of a small block-holder, (e.g. a piece of $\frac{5}{8}$ inch metal rod with multi-grooved end). With the block-holder and adherent paste just above freezing, the tissue block is pushed into the paste with suitable orientation, and at once before time to thaw, the block and holder are plunged into a beaker of petroleum ether which is maintained at low temperature inside a larger beaker containing dry ice and petroleum ether. To mount successfully the block should be cold enough to freeze the brain paste rather than to be itself melted. The block and holder are removed from the cold bath as soon as safely chilled.

2. *Sectioning.* Sections are cut at a constant low temperature in a cryostat such as that described by Møgensen and Linderstrøm-Lang (6). With dry ice as refrigerant thermostatic control is easily accomplished with a small blower actuated by a Fenwal thermal switch. If the front window is made of lucite or plexiglas one inch in thickness, fogging will be largely overcome. Convenience is increased (at a price) with replacement of the dry ice compartment by a refrigeration unit as described by Coons et al. (2). Very light oil is used on the microtome.

Sections 5 to 50 μ are cut one at a time with a slow steady motion and are kept flat by holding a small camel's hair brush against the surface of the block as the section is cut. The brush is moved a trifle faster than the block. The optimal temperature for cutting varies with the tissue, but is usually -15 to -20°C. Too cold, the section may fragment, too warm it may be compressed. The microtome temperature is checked to be sure it has approximately the same temperature as the box, since its temperature will be transmitted to the block.

As cut the sections are transferred serially to a suitable holder with a sharp pointed wooden or plastic stick or forceps tipped with paper or thin flexible plastic. Satisfactory holders (Fig. 1) are made of drilled aluminum blocks sandwiched between glass slides. If the cut sections are nearly as large as the holes, two or more sections can be placed in each hole without mixing the order. As soon as a holder is filled, the cover is replaced to prevent drying of the sections at the temperature of the microtome box, since this would cause shrinkage. One to four section holders are placed in a special glass drying tube (Fig. 1) which has been greased with silicone stopcock grease (Dow Chemical Co.). The tube is transferred, without being allowed to warm up, to another, smaller, constant temperature box kept at -30 to -40°C. After allowing time for the tube and contents to reach the lower temperature the sections are dried by applying a vacuum of 0.1 mm. Hg or less, with a liquid nitrogen or dry ice trap to receive the moisture. Although it is convenient to check the vacuum with a McLeod gauge, it has been thought necessary to have the trap between the gauge and the samples to prevent possible contamination with mercury. Most of the water can be removed in 1 to 6 hours depending on the number and thickness of the sections. The tube is then taken out of the cold box, but evacuation is continued until the tube and contents reach room temperature. The evacuated tube can be safely stored at -25°C. or below for weeks or months, depending on the constituent that is to be measured.

When the vacuum tube is opened to remove samples, the contents must be at room temperature, otherwise moisture will condense on the sections and ruin them. As a precaution the tube is reevacuated before warming, in case the tube may have leaked during storage.

3. *Dissection.* From the dry sections may now be cut the histologically defined volumes for analysis. It would seem desirable to dissect the sections in a room as free as possible of harmful fumes or dust. If the room temperature is above 30°C. myelinated regions become sticky and hard to handle. Cutting is carried out free-hand under a dissecting microscope. The operator wears a mask to prevent blowing away the sections and the clean glass dissecting surface may be

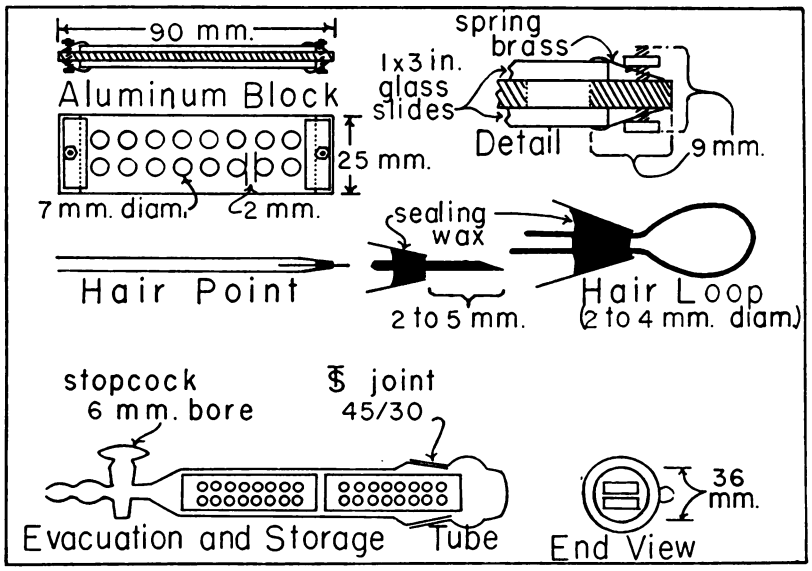

FIG. 1. Hair loop, hair point, aluminum blocks for holding frozen sections and glass evacuation tube for drying frozen sections.

protected from dust by a glass or lucite sheet interposed just below the objective. The sections or fragments are picked up and transferred with a short piece of hair mounted in a glass holder[1] (Fig. 1). Such "hair points" are provided of differing degrees of stiffness for different types of tissue. Suitably chosen they will pick up a tissue fragment without crushing it. During dissection the sections are held down by the left hand with a hair loop, such as commonly used in biological work (Fig. 1). Straight cuts are readily made by pressing down with a scalpel (Bard-Parker blade No. 11). With both hair loop and scalpel held close to the working end, and with fingers resting on the dissecting surface, it is not difficult to cut within 10 or 20μ of the desired point. A fragment 100μ each way is not impractical to dissect out. Such a sample from a 20μ section would have a wet weight of 0.2γ and would contain about 25 packed cells of 20μ diameter.

4. *Histological control.* It usually requires study to recognize sufficient histo-

[1] Suggested by Dr. David E. Smith.

logical detail in the frozen dried material. With familiarity, however, and a good light, a surprising amount of detail can be visualized. If the initial freezing is too slow ice crystals will distort the material and make identification difficult. As needed, sections or fragments of sections are appropriately stained as a guide. For this purpose, as many as 20 or 30 selected sections may be placed in recorded order on a single slide lightly coated with histological glycerine-egg albumin. If wrinkled they are straightened out with two hair loops and then pressed down with the finger tip. They may then be fixed and stained as desired.[2] When necessary the accuracy of dissection may be checked by mounting and staining the unused parts of the section dissected. It is also convenient to place selected dry unfixed sections under a cover slip for study and future reference. The cover slip is sealed at the edges with paraffin and the slides are stored at 4°C. to prevent deterioration.

5. *Measurement of sample size.* The size of the fragment to be analyzed needs to be evaluated. This evaluation has been based on either the protein content, the dry weight, or the sample volume.

The protein is easily measured by a simple procedure which requires no more than 0.2γ protein (10). As a rule protein and at least one other constituent can be measured on each tissue fragment.

The *dry weight* may be determined with either a quartz torsion balance (8) or a quartz "fish-pole" balance (7). In the case of the torsion balance by reducing the volume of the balance chamber, by introducing baffles to decrease air currents and by using a finer suspension a sensitivity of 0.005γ and a reproducibility of 0.02γ has been attained. However, a modified "fish-pole" balance has proven to be much more convenient and is simpler to construct (Fig. 2). The balance illustrated has a sensitivity and reproducibility of 0.01γ. Samples are transported on a glass slide to a position under the balance pan and are transferred to the pan with a suitable hair point while watching through a horizontally mounted dissecting microscope. During the transfer the glass slide serves to support the balance pan and to catch the section if it should fall. Reading may be made at once after closure of the case. The zero point may need frequent small adjustments probably as the result of temperature changes. However, careful temperature control is unnecessary. (Balances of other types which may be suitable are available from the Microchemical Specialties Co., Berkeley, Cal.)

The question arises whether the exceedingly porous tissue may not be so hygroscopic as to give a falsely high dry weight when weighed in room air. To check this point, frozen-dried brain sections were weighed on a fish-pole balance mounted in a vacuum desiccator. After determining the weight *in vacuo*, air of various degrees of humidity was admitted and the new weight measured. The

[2] A convenient rapid Nissl stain is the following adapted from Windle et al (13). The albumin-mounted sections are fixed in 80 per cent ethanol, 5 per cent Formalin, 5 per cent acetic acid for at least 10 minutes. They are rinsed in water and stained 10 minutes in 0.1 per cent thionin in pH 4 acetate buffer (0.025 N sodium acetate, 0.1 N acetic acid). They are placed for 10 seconds in 40 per cent ethanol and 10 seconds in 80 per cent ethanol, each alcohol solution containing 0.1 per cent eosin, and finally passed through absolute ethanol, and 2 butanol changes into xylene.

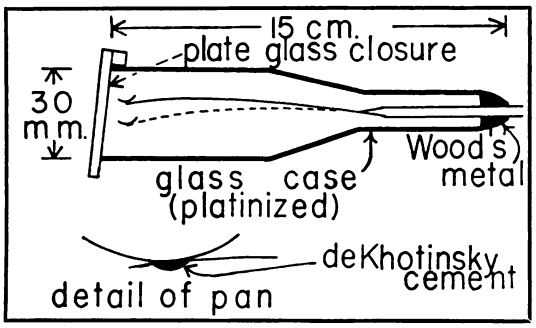

FIG. 2. Fishpole quartz balance with sensitivity about 0.01γ. The inside of the glass case and the fiber are coated with a thin film of platinum (7). Static electrical effects are further minimized by inserting 10 or 20γ of a radium salt suitably painted on a small piece of metal. The pan is made of glass, thin enough to show interference colors, formed by blowing out a glass bubble rapidly. The pan is sealed in place with a minute amount of hard de Khotinsky cement. The speck of cement is first sealed onto the bottom of the pan with heat radiating from a small soldering iron. The pan is then held in position on the fiber with a paper jig and fastened by heat from the soldering iron. Pan and cement for a balance of 0.01 γ sensitivity weigh not more than 5γ. Displacement is measured by reading on a micrometer disk (e.g. 10 mm. in 0.1 mm.) placed in the eyepiece of a horizontally mounted dissecting microscope located in front of the balance. The microscope is adjustable in height (e.g. American Optical Co. model 27K (2× objective, 9× eyepieces)). Samples are placed on the pan with a hair point while observing through the microscope. Weighings may be made at the rate of 1 or 2 per minute. Calibration is effected by weighing single crystals of a suitable colored or fluorescent substance which are then dissolved and measured against appropriate standards.

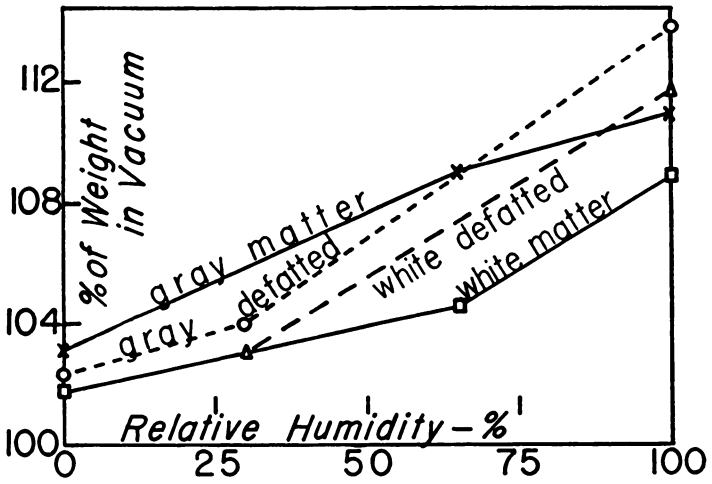

FIG. 3. Effect of moisture and air on the weight of frozen-dried sections of rabbit brain (25°C.).

samples reached each new weight within a few minutes and there was no subsequent drift. It is seen (Fig. 3), that at 25°C. with humidity in the range of 30 to 65 per cent the dry weights are 4 to 9 per cent too high (1 to 2 per cent of the wet weight).

A myelinated sample was slightly less hygroscopic than non-myelinated brain, and, after defatting, the residual (chiefly protein) material was more hygroscopic than the whole sample. It may be of interest that dry air was sufficiently adsorbed by the tissue to account for some of the weight increase (Fig. 3). The weight increase in dry oxygen was almost the same as in dry air.

Although frozen-dried sections weighed in room air tend to adsorb moisture and gases of the air, the increment in weight is consistent, and by making suitable allowance the true dry weight may be estimated to within 2 or 3 per cent. Under usual laboratory conditions a 6 per cent correction is appropriate.

The precision of weighing with the simple balance is illustrated by data on the weight change resulting from alcohol extraction (Table I). Dry sections were extracted in tubes of 3 mm. bore for a few minutes with about 50 cmm. absolute

TABLE I

Reproducibility of weighing with "fish-pole" balance

Frozen-dried samples from several regions of rabbit cortex

Dry Weight					Dry Weight		
Cortical area	Original, γ	After defatting*		Cortical Area	Original, γ	After defatting*	
		γ	Per cent			γ	Per cent
Striate layer I	4.49	2.94	65.4	Insula layer II	2.93	1.93	65.8
	3.42	2.23	65.3		2.33	1.53	65.6
	3.86	2.49	65.5		2.37	1.53	64.4
Striate layer II	3.41	2.27	66.6				
	3.44	2.27	66.0				
	3.15	2.07	65.7				

* Extraction with alcohol and hexane, see text.

ethyl alcohol. The alcohol was removed and the sections were rinsed twice with an equal volume of hexane with care not to let the sections dry until the last. (Otherwise the sections would stick to the tube.)

The *sample volume* can be obtained provided the sections have been cut without serious compression. The volume is calculated from the thickness multiplied by the area. The area is readily measured as follows: The fragment is placed on a microscope slide on a microscope stage under a clean cover slip to keep the sample flat. The monocular eye piece is replaced by a right angled prism, and with a low power objective and bright illumination the image is projected on the wall and traced on a piece of paper. Without moving the microscope a ruled scale with 0.1 mm. divisions (eyepiece micrometer disc) is projected and marked on the same paper. The projected area of the fragment is then measured with a planimeter, using the length of the projected scale to establish the magnification.

The volume of ice at 0°C. is 1.088 times that of water. Brain is about 80 per cent water by weight and has a specific gravity of 1.05. Therefore each ml. of brain contains $.8 \times 1.05 = .84$ ml. water which would be expected to occupy $0.84 \times 1.088 = 0.914$ ml. as ice, an expansion of 0.074 ml. The fat present in

1 ml. brain (0.1 gm.) would contract about .003 ml. on cooling from 38° to −15°C. Thus 1 ml. of brain at 38° would by calculation occupy 1.071 ml. frozen. (The contraction of the tissue solids other than fat from 38°C. to −15°C., and of the ice from 0° to −15° is not over a few parts per thousand and is largely offset by the contraction of the microtome.)

TABLE II

Specific gravity of frozen rabbit tissues relative to water at 25°C.

Tissues frozen in liquid nitrogen unless otherwise indicated

	Specific Gravity		Expansion	
			Observed	Calc.
	25°C.	*−10°C.*	%	%
Grey cortex	1.0506 ± .0014	0.982 ± .002	7.0	7.2
White matter*	1.0493 ± .0006	1.001 ± .002	4.8	5.9
Liver	1.0936 ± .0006	1.0387 ± .0006	5.3	6.4
Liver (frozen slowly)	1.0936 ± .0006	1.0353 ± .0010	5.6	6.4

* Centrum ovale.

TABLE III

Dry weight of cerebral cortex based on volume

Sections of rabbit cortex cut at 20μ. Macro dry weight 216 gm. per kg. or 227 gm. per liter.

Sections Cut at −20°C.				Sections Cut at −30°C.			
Dry* weight	Volume† 10^{-3} μl.	Fresh vol.‡ (calc.) 10^{-3} μl.	Dry weight gm./l.	Dry* weight	Volume† 10^{-3} μl.	Fresh vol.‡ (calc.) 10^{-3} μl.	Dry weight gm./l.
9.32	45.8	42.8	218	10.53	48.4	45.2	233
10.63	50.6	47.3	225	13.23	58.6	54.7	242
13.42	58.8	55.0	244	9.85	44.8	41.9	235
15.39	64.2	60.0	257	9.85	51.2	47.9	205
13.23	64.2	60.0	221	11.56	53.2	49.7	233
12.46	55.0	51.4	243	12.40	52.8	49.3	252
			—				—
Av.			235	Av.			233

* Corrected to weight *in vacuo*.

† Frozen-dried volume calculated from thickness times volume.

‡ Frozen dried volume divided by 1.07, see text.

The above applies only if all the water freezes. To test this, brain and liver were frozen in liquid nitrogen and the specific gravity actually measured in a gradient tube (5, 9) at −10°C. (Table II). Specific gravity standards were provided by K_2SO_4 solutions and methanol-water mixtures which were put into the gradient tube as warm solutions, and read as supercooled droplets which frequently remained unfrozen for many minutes.[3] The specific gravity of the stand-

[3] Even distilled water droplets of 1 to 5 μl. usually remain unfrozen at −20°C. for several minutes, and whole blood droplets do not freeze for 15 minutes or longer.

ards were extrapolated from data for temperatures above zero. It is seen that grey cortex expands about as expected on freezing, whereas white matter and liver—especially when quick frozen—expand about 1 per cent less than expected. This would reasonably be ascribed to incomplete freezing of water dispersed throughout those tissues which are high in total solids. For practical purposes, an expansion of 7 per cent on freezing may be taken for brain.

However, it is necessary to mention a possible source of error in volume measurement with certain tissues, or with tissues frozen or cut in certain ways. For example, with liver very quickly frozen, the sections may be very plastic and have been observed to shrink noticeably in width as they came off the microtome. The dry weight of such liver sections has been found to be as much as 50 per cent too great, indicating that the area had decreased by a third.

To check on the overall validity of the volume measurement for brain, the dry weight of rabbit cortex was determined a) directly on a large sample dried at 105°C. and b) indirectly on small frozen-dried sections for which both dry weight and volume were measured as described above. The average agreement between macro and micro measurements is satisfactory although the indirect values are a few per cent too high (Table III). This is expected from unavoidable compression or wrinkling during cutting. The individual errors are rather large, much larger than the errors in weighing, and are partly ascribable to variations in thickness of cut. Nevertheless the volume measurement is exceedingly useful and the errors are not serious with averages of several samples.

SUMMARY

The general histochemical procedure of Linderstrøm-Lang and Holter has been altered to permit direct histological control and the use of smaller samples. Frozen sections are dried at −30°C. From the dry sections are cut out identified regions as small as 100 x 100 x 20μ (0.2γ wet weight). The chemical analysis of these fragments may be based on either their protein content, their dry weight or their volume. The weight and volume measurements are described and validated. The revised technique is not limited in applicability to the nervous system.

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