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A NEW APPROACH FOR DETERMINING THE VOLUME OF CEREBRAL EXTRACELLULAR FLUID AND DEMONSTRATION OF ITS COMMUNICATION WITH C.S.F.

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SUMMARY

1. A new technique is presented for determining the volume of extracellular space in bowfin (*Amia calva*) brain during *in vitro* incubation. It consists of solving simultaneous equations which are applied to determine the volume of extracellular space as well as intracellular marker concentration. This technique allows for a better insight into the redistribution of marker between incubation medium and extracellular space as well as between extracellular and intracellular space.

2. Na^+, K^+ and Cl^- equilibrated within 10–15 min between incubation medium and extracellular space. There was no evidence of a homoeostatic mechanism controlling the concentration of these ions in the extracellular fluid, which appeared to be in equilibrium with cerebrospinal fluid. The extracellular spaces of these ions were identical: Na^+, 23·4; K^+, 23·3 and Cl^-, 23·2%.

3. Sorbitol equilibrated with the extracellular fluid within 45 min and indicated an extracellular space of 22·6%, nearly identical with that for electrolytes.

4. Vastly different 'spaces' were obtained for [^{3}H]methoxy inulin, which equilibrated within 45 min with a 13% space and [^{14}C]carboxyl inulin, which showed a 46% space value for only 30 min. The difference may be explained by marker decomposition. The 9% difference between the [^{3}H]methoxy inulin and sorbitol spaces may be explained by a 'packing' factor attributable to molecular size.

INTRODUCTION

Estimates of the volume of extracellular space in the central nervous system vary over a wide range. Data from *in vivo* experiments were based on the assumptions that the composition of the extracellular fluid resembles either blood plasma or cerebrospinal fluid. The extent to which these assumptions affect the estimates was shown by van Harreveld (1966) who suggested that an extracellular space in the order of 20 % is obtained if the values for chloride space (31·4 %, Davson, 1956), sulphate space (4 %, Timaras, Koch & Ballard, 1956; Barlow, Domek, Goldberg & Roth, 1961) and thiocyanate spaces (4–17 %, Streicher, 1961) based on plasma concentrations are recalculated on the basis of cerebrospinal fluid concentrations. Also, a 'sink action' is provided by the constant circulation of the cerebrospinal fluid, as determined from data based on ventriculocisternal perfusion (Oldendorf, 1967; Oldendorf & Davson, 1967; Davson, 1967). Estimates of very small (3–5 %) extracellular spaces in electron micrographs of mammalian brains (Horstman & Meves, 1959) may be subject to error due to rapid volume changes of cells upon, or after, death, plus the effects of fixation, dehydration and embedding. The extracellular spaces were considerably larger in electron micrographs of brains frozen *in situ* (van Harreveld, Crowell & Malhotra, 1965; Malhotra & van Harreveld, 1966; van Harreveld & Malhotra, 1967). Estimates based on tissue slices *in vitro* are affected by damage to cut surfaces, facilitating the entrance of marker into the intracellular compartment, and by changes induced by the swelling of tissue.

The present investigation concerns the distribution of various markers, including neutral molecules and electrolytes, between an artificial c.s.f. and preparations of fish brains *in vitro*. This technique is advantageous in eliminating 'sink actions' by precise control of the composition of the incubation medium; previous studies have shown that these *in vitro* brains resemble their *in vivo* state in terms of fine structure and electrolyte content during 30 min incubation (Friede, Hu & Cechner, 1969). Five different markers were tested, and a new method of calculation based on solving simultaneous equations is introduced. The values for the size of the extracellular compartment were identical, except for inulin.

METHODS

The bowfin (*Amia calva*), a primitive fresh-water fish, was chosen for previous experiments on glia cells and their footplates; its brain is well suited for *in vitro* experiments because of its resistance to post mortem deterioration. The resistance may be due to a slow metabolic rate and a low body temperature. Also, the brain is approximately 1–1·5 mm thick for most parts and sparsely vascularized. Hence, the

transition to a supply of nutrients from the surface of the brain may be less traumatic than for mammalian brain; in cat brain, for example, the area for diffusion from intracerebral blood vessels is more than 10 times greater than that from the brain surface (Coulter, 1958).

Freshly caught bowfin were stored in an aerated tank at a controlled temperature of $16 \pm 1°$ C; all experiments were performed within 3 days of shipment. The brains were removed, washed in chilled artificial cerebrospinal fluid containing normal concentrations of electrolytes and glucose and were cleaned of adhering blood and excessive meningeal tissue. The choroid plexus covering the fourth ventricle and hemispheres were removed, providing a wide communication between the ventricles and incubation fluid. The composition of 'normal' artificial cerebrospinal fluid was (in m-equiv/l.): Na^+, 145; K^+, 5; Mg^{2+}, 2; Cl^-, 130; HCO_3^-, 20; SO_4^{2-}, 2; glucose, 5 mM; saturated before use with 95 % O_2 and 5 % CO_2. For variation of Na^+ and Cl^-, sodium acetate and choline chloride were used. The desired amount of 'marker' was added to normal c.s.f. as specified in the Tables. Incubation temperature was 20° C.

After incubation, the brains were rinsed for 1 sec in deionized water to remove the external film of incubation fluid, sliced to open all ventricles and blotted completely to remove any fluid trapped in the ventricles or meninges. The brains were weighed and homogenized.

All chemicals used were Baker Analyzed grade. ^{36}Chloride (HCl), [^{14}C]carboxyl inulin and [^{3}H]methoxy inulin (sample *a*) were purchased from International Chemical and Nuclear Corp. (Irvine, Calif.), [^{3}H]methoxy inulin (sample *b*) from New England Nuclear (Boston, Mass.), [^{3}H]L-sorbitol from the Radiochemical Centre (Amersham/Searle, Arlington Heights, Ill.).

Sodium and potassium were determined by flame photometry with a double-channel photometer (Model 142, Instrumentation Laboratory, Inc., Boston, Mass.). Internal lithium standard was used. All samples were measured in duplicate with concurrent standard solution. The analytical error was within $\pm 0{\cdot}2$ m-equiv/l.

Total chloride was determined by titration with mercuric nitrate (Hu & Friede, 1971), except that a deproteinized tissue homogenate was used to improve the end-point of chloride titration.

Isotope markers were determined by liquid scintillation counting with a Unilux T.M.II liquid scintillation system. To eliminate error due to the enhancing or quenching effect of the matrix of fish tissue, an equal quantity of control (non-labelled) fish brain homogenate was added to the incubation solution; for counting the samples were diluted to the same range of radioactivity as the incubated fish brain homogenate. This method of compensation for the enhancing or depressing effects of a tissue matrix was introduced earlier for atomic absorption spectrophotometric measurements (Hu & Friede, 1968).

For determination of water content (% of wet weight) the brains were prepared as for chemical analysis, weighed immediately in covered crucibles and dried to constant weight at 100° C. The drying process was repeated at 105° C.

Calculation

(*a*) *By simultaneous equations.* Simultaneous equations are equations which must be satisfied simultaneously by the same values for the unknowns. For solving two unknowns, two equations with two sets of data are required:

Let

C = intracellular sodium content (m-equiv/kg of tissue),

X = extracellular volume of brain (fraction),

$(1-X)$ = intracellular compartment of brain.

$C(1-X)+$(concn. of Na^+ in incubation solution) X = total Na^+ content of brain. For example (Table 1):

$$\begin{aligned} (1)\quad & C(1-X)+162X = 54{\cdot}7, \\ (2)\quad & C(1-X)+128X = 46{\cdot}7, \\ (3)\quad & C(1-X)+\ 95X = 39{\cdot}0, \\ (1)-(2)\quad & \qquad 34X = 8{\cdot}0, \\ & \qquad\ \ X = 0{\cdot}23. \end{aligned}$$

Intracellular marker content is calculated by substituting the value for extracellular space into the above equations. For instance, intracellular sodium content, C, is calculated by substituting the sodium space, X, with 0·23 and solve for C.

(*b*) *By fraction of marker concentration.* Extracellular space was calculated from the concentration of isotope in the brain expressed as a fraction of its concentration in the medium:

$$\text{Chloride space} = \frac{\text{cpm/g tissue}}{\text{cpm/ml. of incubation solution}}.$$

Space (%) = fraction × 100.

RESULTS

Water content. The water content of the brains (Fig. 1) was determined for incubation in normal artificial cerebrospinal fluid and in hypo- and hypertonic solutions which covered the range of osmolarities used for all experiments. Each value is an average of data from three brains, the control for four. These determinations were accurate within 0·1 % as the wet weight of the brains was about 400 mg and the sensitivity of the balance 0·1 mg. The sum of experimental errors and variation among brains was within 0·5 %.

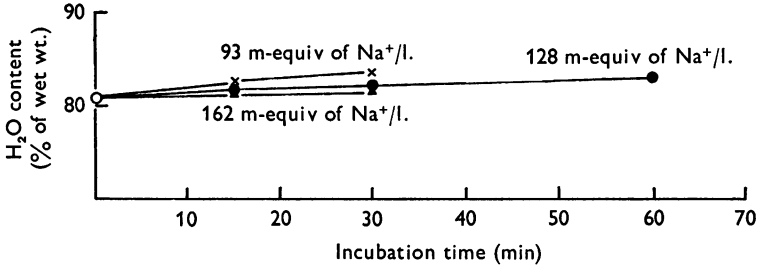

Fig. 1. Changes in water content of bowfin brain during incubation *in vitro*.

Brains incubated for 1 hr in normal cerebrospinal fluid showed a 1·6 % increase in water content. Water content was constant during 30 min incubation in slightly hypertonic solution (162 m-equiv Na), and the increase was somewhat greater than normal in hypotonic solution (95 m-equiv Na).

The increase in water content did not affect the values of the extracellular space when calculated by the simultaneous equations method. For

these calculations, the effect of variation in water content was cancelled out because the values for extracellular space were based on the differentials of the marker concentrations for equal incubation times. For calculations based on the fraction of marker concentration, the error resulting from the increasing water content varied with incubation; it approximated 0·2 % for 10 min incubation in decreased Na^+ content, or 0·4 % for 1 hr in normal artificial cerebrospinal fluid. van Harreveld, Collewijn & Malhotra (1966) observed no change in the size of extracellular compartment of hydrated rabbit brains.

TABLE 1. Sodium space. Each value is an average of data from two fish

	Experimental data			Calculated values	
Incubation period (min)	Incubation solution Na^+ (m-equiv/l.)	K^+	Whole brain Na^+ content (m-equiv/kg)	Extracellular space (fraction)	Intracellular Na^+ content (m-equiv/kg)
10	162	3	54·7 (1)	(1–2) 0·235	21·9
	128	3	46·7 (2)	(1–3) 0·234	21·9
	95	3	39·0 (3)	(2–3) 0·233	21·9
				Av. 0·234 ± 0·001 (S.D. of mean)	
20	162	3	54·6 (1)	(1–2) 0·218	21·2
	128	3	47·2 (2)	(2–3) 0·260	22·1
	95	3	38·6 (3)	(1–3) 0·234	21·1
				Av. 0·237 ± 0·015 (S.D. of mean)	
30	162	3	55·0 (1)	(1–2) 0·288	7·8
	128	3	45·3 (2)	(2–3) 0·318	8·8
	95	3	34·8 (3)	(1–3) 0·302	8·3

Sodium space. Incubation of brains in Na^+, 95, 128 and 162 m-equiv/l., resulted in corresponding increases or decreases in total Na^+ content (Table 1). The values were in a similar range for 10 and 20 min incubation, with slightly decreased Na^+ after 20 min in 95 m-equiv/l. and slightly increased Na^+ in 162 m-equiv/l. These changes were more pronounced after 30 min, indicating that redistribution of Na^+ commenced by 20 min, the direction of the flow depending on the concentration of Na^+ in the incubation medium.

Calculations with the simultaneous equations method gave constant values for the extracellular space (23·4 ± 0·1 S.D. of mean) and for the intracellular Na^+ content (21·9 m-equiv/l.) for 10 min incubation. These data confirm the veracity of the assumptions on which the calculations are based, namely: (1) that the incubation fluid equilibrates with the extra-

cellular compartment and (2) that the sodium concentration remains constant for the intracellular space. These conditions were present for 10 min incubation. The Na^+ of the incubation fluid began to equilibrate with the intracellular compartment after 20 min, the direction of the flow depending on the ionic strength of the external fluid (Table 1). The intracellular electrolyte equilibrium became disturbed, and the redistribution of Na^+ was no longer representative of the size of the extracellular compartment.

TABLE 2. Potassium space. *Each value is an average of data from three fish; the rest is an average of two fish

	Experimental data			Calculated values	
Incubation period (min)	Incubation solution Na^+ (m-equiv/l.)	Incubation solution K^+ (m-equiv/l.)	Whole brain K^+ content (m-equiv/kg)	Extracellular space (fraction)	Intracellular K^+ content (m-equiv/kg)
10	128	30	83·9 (1)	(1–2) 0·233	100·9
	128	15	80·4 (2)	—	101·2
20	122	31·0	*81·8 (1)	(1–2) 0·239	96·0
	132	15·1	*78·0 (2)	(2–3) 0·200	95·6
	144	5·1	*76·0 (3)	(1–3) 0·224	96·2
				Av. 0·221	
30	123	32·5	*80·5 (1)	(1–2) 0·231	94·0
	135	15·2	*76·5 (2)	(2–3) 0·202	93·6
	145	5·3	*74·5 (3)	(1–3) 0·221	93·8
				Av. 0·218	

Potassium space. The potassium content of brains incubated in fluids containing K^+, 5–30 m-equiv/l., are shown in Table 2. The data show a slight, progressive decrease in the total K^+ content of the brains with increasing incubation periods, paralleled by a similar decrease in the calculated values for intracellular K^+ content. The values for intracellular K^+ were constant for each given incubation period, and the values for extracellular space were constant throughout.

The data indicate that K^+, just as Na^+, takes only 10 min to establish equilibrium between extracellular and cerebrospinal fluid. The size of the compartment (23 %) also was alike for K^+ and Na^+. During incubation the brains lose K^+ progressively from the intracellular compartment, the loss being slightly less when K^+ content of the extracellular space is elevated (Table 2). Our data are consistent with the findings that isolated mammalian nerve and glia cells maintain constant intracellular K^+ for 25 min when incubated at 4° C (Hamberger & Rockert, 1964), which is 16 degrees lower than the present series.

TABLE 3. Chloride space. Each value is an average of data from three fish

Incubation period (min)	Experimental data: Incubation solution: ^{36}Cl (counts/min.ml.)	Experimental data: Incubation solution: Cl^- (m-equiv/l.)	Experimental data: Whole brain: ^{36}Cl content (counts/min.g)	Experimental data: Whole brain: Cl^- content (m-equiv/kg)	Calculated values: Extracellular space: from ^{36}Cl activity	Calculated values: Extracellular space: from Cl^- content	Calculated values: Intracellular Cl^- content (m-equiv/kg)
5	86,440	110	15,847	46·7 (*a*)	0·183	(*b*–*a*) 0·041	26·4
	130,190	144	30,343	48·1 (*b*)	0·233	(*c*–*b*) 0·237	18·3
	156,000	182	34,313	57·1 (*c*)	0·220	(*c*–*a*) 0·145	18·3
30	83,200	110	19,240	38·9 (*d*)	0·231	(*e*–*d*) 0·232	17·4
	130,520	144	30,107	46·8 (*e*)	0·231	(*f*–*e*) 0·334	17·5
	160,180	182	42,583	69·4 (*f*)	0·266	(*f*–*d*) 0·428	35·3

Chloride space. Data on chloride space were obtained simultaneously from the distribution of isotopic tracer (^{36}Cl) and from chemical analysis of total chloride content of the same brains. This permitted much better insight into the interplay of the distribution of Cl^- between incubation medium and extracellular space, as well as between extracellular space and cells. For media containing normal concentration of Cl^- (144 m-equiv/l.), the isotope tracer (^{36}Cl) established equilibrium within 15 min, maintaining it for 30 min. The chloride space was 23 % and the total chloride content of the brain decreased only slightly (2·6 %) during 30 min incubation.

For solutions in which the Cl^- content was lower or higher than normal, the rate of equilibration was modified by additional exchanges of chloride between the brain and the medium. Equilibration in low chloride (110 m-equiv/l.) was delayed for up to 30 min as it occurred against an outflow of chloride from the tissue into the incubation medium, amounting to a loss of 7·8 m-equiv/l. (column 4 of Table 3). For solutions with higher than normal Cl^- content (182 m-equiv/l.) equilibration was attained in 15 min, being superimposed by a continuous influx of Cl^- into the tissue.

Careful analysis of the data in Table 3, thus, indicates that Cl^- equilibrated with a 23·3 % space. In addition, the intracellular Cl^- content depends much more on the extracellular fluid than either Na^+ or K^+, the direction of the flux being determined by the concentration gradients between incubation fluid and brain.

Sorbitol space. Sorbitol required longer incubation periods (45 min) than the electrolytic markers to reach equilibrium between cerebrospinal and extracellular fluid (Fig. 2). This longer period may be attributable to the molecular size of sorbitol being much larger than that of the electrolytes. The extracellular spaces calculated by the fraction of marker method (22·1 %) and by simultaneous equations method (22·6 %) were in excellent agreement for 45 and 60 min incubation (Table 4). These values were 1 % lower than those for electrolyte markers, a difference which may also be explained by the size of the marker molecule (Discussion).

Inulin space. The distribution of inulin was studied for three different samples: [^{14}C]carboxyl inulin and two samples of [^{3}H]methoxy inulin from different manufacturers. The diffusion of inulin from the incubation medium to extracellular space reached equilibrium in 45 min, the same as sorbitol. The two samples of [^{3}H]methoxy inulin gave identical space values of 13 % for 45 min incubation; accordingly, only one curve is shown in Fig. 2. A much larger space value (46 %) was obtained with [^{14}C]-carboxyl inulin after only 30 min incubation. All values for different markers and incubation periods are shown in Fig. 2.

The 9 % difference between sorbitol space and the [^{3}H]methoxy inulin space may be explained by the difference in the molecular weight (180 and

5,000–5,500) of these markers (Discussion). The vast difference in the space volume (13 and 46 %) of the same compound (inulin when different radicals are labelled, i.e. methoxy- and carboxyl-) can be explained best by a chemical decomposition theory, the decomposition taking place at the ^{14}C bond. Consequently, there are two different isotopic species present in the fluid, the inulin and the decomposed product which is permeable to the cells; hence, the resultant extracellular space is more than three times the 13 % methoxy inulin space.

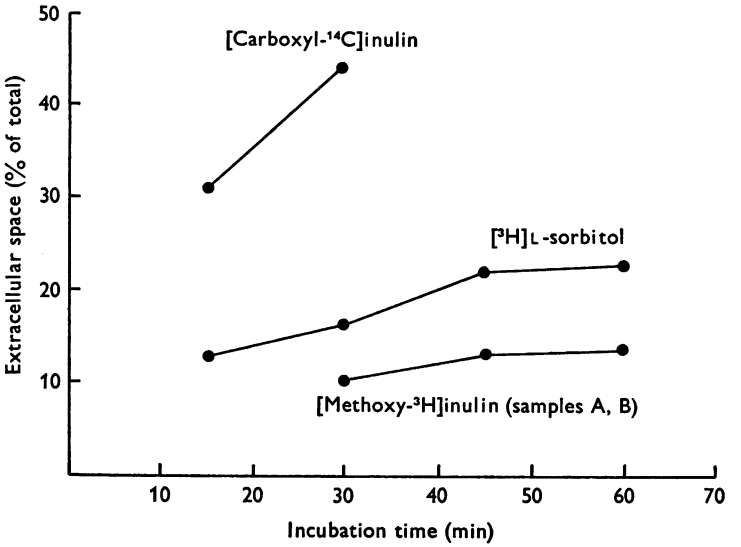

Fig. 2. Distribution of sorbitol and of three samples of inulin in bowfin brain during incubation *in vitro*.

TABLE 4. Sorbitol space. Each value is an average of data from three fish

	Experimental data		Calculated values: Extracellular space (fraction)	
Incubation period (min)	Incubation solution (counts/min.ml.)	Whole brain (counts/min.g)	from fraction method	from simultaneous equations
45	2,818,856	608,053 (1)	0·216	(2–1) 0·208
	6,116,813	1,293,674 (2)	0·212	(3–2) 0·234
	8,619,131	1,878,093 (3)	0·214	(3–1) 0·220
				Av. 0·221
60	2,868,294	639,017 (4)	0·223	(5–4) 0·227
	6,200,813	1,394,149 (5)	0·222	(6–5) 0·216
	8,476,519	1,885,091 (6)	0·219	(6–4) 0·228
				Av. 0·226

DISCUSSION

Preparations of small, intact brains for *in vitro* studies of volume and/or composition of the fluids in the extracellular compartment offer the evident advantages of eliminating the need for stabilizing and monitoring the reference fluids cerebrospinal fluid and/or blood. The distribution of markers between incubation fluid and brain is confined to two transfer processes: incubation fluid with extracellular compartment, and extracellular with intracellular compartment.

The advantages of small intact brains over brain slices are evident: most portions of bowfin brain are about twice as thick as is customary for brain slices (0·5 mm) and tissue trauma is almost completely eliminated. Consequently, there are striking differences in the degree of swelling, which was about 1·6 % in 1 hr for bowfin brain, while mammalian brain slices incubated aerobically in chilled media swell 19 % during 5 min and 31 % during 60 min incubation. Extracellular space markers do not penetrate such brain slices until they are swollen, the penetration being limited to their superficial, damaged portion (Pappius & Elliott, 1956; Pappius, Klatzo & Elliott, 1962).

The present data on bowfin brain are extremely similar to those by Bradbury, Villamil & Kleeman (1968) for the brain of *Rana pipiens* which in its size and its stability during *in vitro* incubation appears to resemble bowfin brain. Swelling of frog brains during 1 hr incubation was 2·2 %. The Na^+ and Cl^- spaces for 10 min incubation were 23·8 and 22·7 %, and the sucrose and inulin spaces for 30 min were 22·1 and 15·8 %; all these values are in close agreement to the electrolyte, sorbitol and inulin spaces in the present study, and to the 24·1 % Na^+ space and 16·2 % inulin space of frog brain reported by Zadunaisky & Curran (1963).

The potential of the present experiments was enhanced by the newly introduced method of calculation by solving simultaneous equations. The consistency of the values obtained for extracellular space and intracellular marker concentration demonstrates the reliability of the method. The calculated results indicate excellent agreement for Na^+ and K^+ spaces of 23 %. Cl^- equilibrates with a space of the same volume, but its further redistribution between the extracellular fluid and the cells is much greater than for Na^+ or K^+. The extracellular space determined by electrolyte markers was within 1 % of the value of the sorbitol space. The extracellular compartment of bowfin brain, therefore, may be determined with considerable confidence and accuracy as being 22–23 %.

Each of the markers showed specific characteristics in terms of time for equilibration between incubation fluid and extracellular fluid, and in terms of its facility in entering the intracellular compartment. Values for extra-

cellular space may be too low due to insufficient time for equilibration with the extracellular compartment and due to other technical factors discussed in Material and Methods. Comparison of Tables 1, 2 and 3 indicates that entrance of a marker into cells is not necessarily an indication that the cells deteriorate; for example, Cl^- redistributed much more freely than either K^+ or Na^+. Hence, the 'chloride compartments' of more than 30 % reported by numerous investigators cannot be related to specific anatomical compartments nor to tissue deterioration. It is also questionable whether increases in 'marker spaces' for prolonged incubation periods may be attributed to the break-down of specific cell populations, as suggested by Vernadakis & Woodbury (1965). The footplates of the ependymoglia of bowfin, for example, swell much more rapidly and extensively than their perikarya (Friede *et al.* 1969), and there is reason to assume that the same applies to synaptic terminals of dendrites as compared with nerve cell bodies (Cornog, Gonatas & Feierman, 1967). Our observations support Pollay & Kaplan's (1971) conclusions for rabbit brain that there is only one extracellular space; the description on 'special spaces' for various markers is misleading when applied to anatomically defined tissue compartments or cell populations.

The data obtained for inulin are of considerable interest. The 'spaces' obtained for carboxyl-labelled inulin were more than three times those for methoxy-labelled inulin, and much less constant. This was considered the result of rapid decomposition of the carboxyl bond during incubation, the decomposed products having diffusion characteristics different from the original labelled molecule. Similar very large 'inulin spaces' [^{14}C]carboxyl were reported by Levi (1969) (42·7 %, New England Nuclear; 57 %, Calbiochem.) and by Levi & Lattes (1970). Cohen, Blasberg, Levi & Lajtha (1968) and Cohen, Stapleman & Lajtha (1970) demonstrate the dependence of these large [^{14}C]carboxyl inulin spaces on temperature (49 % at 0° C, 62 % at 37° C). Nicholls & Wolfe (1967) present scintillation counts and radioautographic data indicating the entrance of [^{14}C]carboxyl inulin into nerve cells, but no indication for penetration of [^{3}H]methoxy inulin into nerve cells was found by Brown, Stumpf & Roth (1969). Ferguson & Woodbury (1969) report an [^{14}C]inulin uptake ratio of 58 % for adult rat brain and cerebrospinal fluid. On the basis of the present data, such large 'spaces' should be considered with reservation; evidently they are of little significance for determining the size of anatomically defined tissue compartments.

Methoxy-labelled inulin showed equilibration with extracellular space, yielding a much smaller 'inulin space' of 13 %. A similar difference between the ^{24}Na spaces (24·1 and 23·6 %) and the inulin spaces (16·2 and 18·6 %) was reported by Zadunaisky & Curran (1963) and Bradbury *et al.*

(1968) for frog brains *in vitro*. Also, many other studies of inulin spaces in mammalian brains report similar low values when compared with other markers (Varon & McIlwain, 1961; Woodward, Reed & Woodburch, 1967; Hertz, Schousboe & Weiss, 1970).

Several explanations for the 9 % difference between inulin and sorbitol spaces in our data may be considered: first, there may be no equilibration for the central core of large brains, even for prolonged incubation periods. In rabbit brain, the penetration of inulin from the ventricles into the tissue diminishes rapidly with increasing depth (Rall, Oppelt & Patlak, 1962); extrapolation to the region of equilibrium indicates a space of 12 %, that is within 1 % of our value. In bowfin brain few zones are more than 0·5 to 1 mm off a surface exposed to the incubation, and equilibrium is readily attained. Secondly, inulin may not be able to enter compartments into which sorbitol and electrolytes enter. Yet, it is difficult to conceive a type of cell, or cell process, in the brain having this characteristic. The lumen of the vascular compartment may qualify for an inaccessible space of 9 %, but it is unlikely that inulin should not enter the blood vessels which it is known to leave without difficulty. The blood content of petromycon brain, which is somewhat lower than bowfin phylogenetically, is 4–5 % of weight, of which 57 % is in choroid plexus (Heisley, 1968). As the third and most likely explanation, we propose that the 'inulin space' is smaller due to a molecular 'packing effect'. The width of cerebral extracellular spaces is quite sufficient to admit the inulin molecule; the narrowness and angulations of these spaces, however, may suffice to reduce the density of molecular packing of the large inulin molecule with a molecular weight of 5,000–5,500, similar to suggestions by Cohen *et al.* (1970). These authors postulate the existence of a system of thin channels into the interior of the cells, which are incompletely filled by marker molecules of large size. However, there is no support for the existence of such a system in electron microscopy. Another more likely explanation may be sought in the carbohydrate or glycoprotein coating of the plasma membrane of cells (Rambourg & Leblond, 1967). The presence of such a 'fuzz' within the narrow cerebral extracellular spaces (Revel & Ito, 1967) may well affect the packing of a large molecular marker, explaining the differences found in our data for electrolyte markers (23 %), sorbitol (22 %) and inulin (13 %). This concept is amenable to further testing using markers of greater variety in molecular sizes. It may also explain why [^{3}H]inulin and [^{14}C]sorbitol spaces did not show similar differences for frog muscle (28·1 % and 25·4 % respectively; Dryden & Manery, 1970), where extracellular spaces are much wider. These considerations indicate a need for reappraising the usefulness of inulin for accurate determinations of cerebral extracellular space; inulin values may be mere aliquots of the actual extracellular volumes.

The surface across which the marker exchanges take place needs to be discussed. Several authors considered the ependyma a barrier capable of active, selective transport of substances (Feldberg & Fleischhauer, 1960; Edstrøm & Steinwall, 1961). However, their conclusions were based on the cessation of exchanges post mortem, which are, more likely, indicative of the obliteration of the extracellular compartment by the post mortem swelling of the cells (Rall *et al.* 1962; Davson, 1967). These conclusions also are inconsistent with the demonstration of the *in vivo* penetration of peroxidase from ventricles into the tissue along the extracellular clefts (Brightmann, 1965). High permeability of the ependyma has been demonstrated for ^{131}I (Bito, Davson, Levin, Murray & Snider, 1966) and ^{42}K (Domer & Whitcomb, 1964; Bradbury & Davson, 1965; Katzman, Graziani, Kaplan & Escriva, 1965; Cserr, 1965). The flux of ^{24}Na across ependyma is about 400 times greater than that across capillaries, which are considered a highly permeable system (Davson & Pollay, 1963). In bowfin brain the meningeal surface possesses much fewer intercellular junctional complexes than the ependymal surface (Friede, Hu & Johnstone, 1969); hence, penetration through the meningeal surface should meet even less resistance than through the ependyma. Accordingly, we concluded that the exchanges between incubation fluid and brain took place through both the meningeal and ependymal surfaces, the extracellular fluid being in free communication with cerebrospinal fluid throughout.

These observations confirm the concept that the cerebral extracellular fluid communicates and exchanges with cerebrospinal fluid, as proposed by Wallace & Brodie (1939). The only limitation imposed on the exchange of electrolytes is the length of the diffusion path which depends on the size of the brain. Hitherto, experimental support for this concept was indirect; it was based on the exchanges between brain and ventriculo-cisternal perfusion fluids (Davson, 1958, 1967) and on the responses of respiratory neurones to changes in the ionic composition of the cerebrospinal fluid (Fencl, Miller & Pappenheimer, 1966). Rapid exchanges of Na^+ and K^+ between incubation fluid and brain tissue was demonstrated by Nicholls & Kuffler (1964), Kuffler, Nicholls & Orkand (1966) and Kuffler & Nicholls (1966). The present data indicate that bowfin brain does not possess homoeostatic systems by which the extracellular concentrations of Na^+ and K^+ is stabilized in a narrow range (Cohen, Gerschenfeld & Kuffler, 1968). They provide no evidence to support the concepts that glia cells may actively control the concentration of Na^+ (Katzman, 1961; Friede, 1964) or of K^+ in the extracellular compartment (Orkand, Nicholls & Kuffler, 1966; Baylor & Nicholls, 1969). The data in Tables 1 and 2 could not have been obtained were such control systems present and active. There is no support, thus, for the hypothesis that glia cells play a significant role in controlling

the microenvironment of the neurones in terms of controlling the concentration of the major cations in the extracellular fluid.

Note added in proof

A recent report by Marlow & Shephard (1970) shows that [^{14}C]carboxyl inulin is a poor tracer of native inulin in a physiological system and that [^{3}H]methoxy inulin and inlin-T are better tracers.

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