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DISTRIBUTION OF INULIN, SUCROSE AND MANNITOL IN RAT BRAIN CORTEX SLICES FOLLOWING *IN VIVO* OR *IN VITRO* EQUILIBRATION

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SUMMARY

1. The distribution spaces of inulin, sucrose and mannitol in rat brain cortex slices were determined in tracer experiments. Tracer equilibration times of 5 hr were used with combined ventriculo-cisternal perfusion and I.V. infusion of tracers. After 5 hr of equilibration the distribution volumes (expressed as μl./100 mg wet wt.) obtained using the perfusion fluid as reference and after correction of tracer amount in residual blood were obtained. Information on cellular uptake was obtained by examining a slow component of wash-out curves from brain slices after *in vivo* equilibration. Distribution spaces were corrected for intracellular penetration to obtain the extracellular volumes of distribution. Inulin was found to equilibrate with 13·3 μl. extracellular tissue water per 100 mg initial wet weight, sucrose with 16·6 μl. and mannitol with 19·5 μl.

2. Marker spaces in rat brain cortex slices were also analysed by compartmental analysis of efflux of tracer inulin, sucrose and mannitol following *in vitro* incubation in a balanced medium for 60 min. Following *in vitro* equilibration, inulin was found to equilibrate with 22·4 μl. extracellular tissue water per 100 mg of final wet weight, sucrose to equilibrate with 31·7 μl. extracellular tissue water and mannitol with 42·3 μl.

3. Inulin space *in vitro* when allowance is made for swelling produced during incubation of the slice was similar to the chemically delineated extracellular distribution volume *in vivo*. Sucrose and mannitol spaces *in vitro*, however, did not accord with *in vivo* determinations, most likely due to access of markers to areas of cell damage in the slice arisen during incubation.

4. Comparison of fluid spaces delineated by inulin in slices of rat cerebral cortex following *in vivo* and *in vitro* equilibration indicated that swelling produced during incubation *in vitro* mainly occurred in the extracellular space. Swollen glial cells may account for the small, but significant intracellular swelling in incubated slices.

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INTRODUCTION

A fluid compartment can be estimated in histological sections of a tissue and defined on a morphological basis (van Harreveld, Crowell & Malhotra, 1965; van Harreveld & Malhotra, 1966) or it can be estimated as the apparent volume of distribution for a particular solute when true equilibrium is achieved. The extracellular space of a tissue represents distribution volume for solutes completely excluded from the intracellular compartment. Inulin, sucrose and mannitol have been employed for the purpose of defining the extracellular space on a chemical basis. The ambiguity of the term extracellular space, however, becomes evident from the fact that small marker molecules appear to have larger distribution volumes than big molecules. The difference in extracellular space when measured with markers with different molecular weight can be due to different degree of permeation of the markers into an intracellular compartment during equilibration, or merely reflect that some fraction of the extracellular fluid is unaccessible to marker molecules due to steric hindrance (van Harreveld & Crowell, 1969).

In vitro approaches to the delineation of compartments in neuronal tissue allow experiment to be performed under controlled conditions. Many studies of volumes of distribution in brain tissue have been carried out on isolated brain slices exposed to radioactive markers during *in vitro* incubation (Pappius, 1965; Cohen, Blasberg, Levi & Laytha, 1968; Lund-Andersen, 1974). In this preparation the intra- and extracellular compartments could be better separated than by use of whole brain preparations exposed during *in vivo* experiments.

The present paper deals with distribution in rat brain cortex slices of some commonly used extracellular markers exposed to brains during long-term ventriculocisternal perfusion and intravenous infusion. Furthermore, the present examination analyses the *in vitro* situation in which brain cortex slices were exposed to the same extracellular markers during 60 min of incubation with subsequent determination of the efflux of markers as described by Lund-Andersen (1974).

METHODS

Male Sprague-Dawley rats weighing 300–320 g were used.

In vivo experiments

Preparation of animals. Anaesthezia was introduced by I.P. injection of pentobarbitone (50 mg/kg) and further maintained by injections of pentobarbitone I.V. as required. Both kidneys were exposed by a retroperitoneal approach and the animals were functionally nephrectomized. Tracheostomy and intubation was carried out and polyethylene catheters were introduced into a femoral artery and vein. Arterial blood samples were drawn into heparinized glass capillaries for blood gas and isotope determinations. The arterial blood pressure was continuously recorded by means of a transducer. Rectal temperature was maintained at 37·8–38·2 °C by means of a heating pad. The animals were immobilized with gallamonium iodide (20 mg/kg) I.V. Artificial respiration was given with a small animal respirator.

Ventriculo-cisternal perfusion. Two inflow cannulae (0·35 mm o.d.) were placed, one in each lateral ventricle. The ventricular system of the brain was perfused with artificial cerebrospinal fluid. The cannulae were connected to a slow infusion pump (Braun-Melsungen). The rate of infusion was 15·1 μl./min evenly distributed upon the two lateral ventricles. The artificial

cerebrospinal fluid contained (mM): NaCl 126, $NaHCO_3$ 22, Na_2HPO_4 1, KCl 2·8, $MgCl_2$ 0·88, $CaCl_2$ 1·45, glucose 3·5. The solution was equilibrated with 5 % CO_2 to give a final pH of 7·4. The osmolarity of the solution was 289 m-osmole. The atlanto-occipital membrane was exposed and a cisternal cannula was inserted into the cisterna magna; outflow fluid was collected via polyethylene tubing into preweighed glass vials at a pressure of − 10 cm water.

Each animal received 25 μc [^{3}H]mannitol, 10 μc [^{14}C]sucrose or 10 μc [^{14}C]inulin I.V. The level of the isotopes in blood plasma was kept constant by frequent I.V. injections of decreasing amounts during the experimental period. The patterns of tracer administration required to keep plasma concentrations approximately constant were determined in preliminary experiments on rats with ligated renal vessels. Sufficient [^{3}H]mannitol, [^{14}C]sucrose or [^{14}C]inulin was added to the perfusion fluid to make the perfusate and plasma concentrations of the isotopes equal. Perfusate was sampled from the cisterna magna every 15 min and samples of arterial blood were taken every 30 min. After 5 hr of attempted steady-state perfusion the animals were killed by decapitation and exsanguinated to minimize the vascular blood content of the brain. The brain was rapidly removed, the cerebral cortex was separated by peeling off subcortical tissues. Areas of cortex through which the inflow needles were introduced into the lateral ventricles were discarded. The posterior part of the left hemisphere was taken for analysis *in toto*. The right posterior part of the cortex was cut in slices from the lateral surface of the hemisphere and inward towards the ventricular surface. The thickness of a slice was 0·5–0·6 mm and the number of slices was 5.

In separate experiments the volume of residual blood in brain tissue was estimated using ^{113m}Indium, which is bound to plasma transferrin as a plasma tracer (Hossain, McIntyre, Poulose, Stern & Wagner, 1969). Freshly generated ^{113m}Indium was injected I.V. 5 min before the end of the experiment. Approximately 500 μc was injected.

In vitro experiments

Anaesthezia was introduced with pentobarbitone and the animals were killed by decapitation. The skull was opened and the brain rapidly removed. The brain was divided and brain slices from posterior part of each hemisphere prepared. After preparation the slices were incubated for 60 min in 4 ml. medium containing [^{14}C]inulin 1 μc, [^{14}C]sucrose 1 μc, or [^{14}C]mannitol 1 μc. The composition of the incubation medium was equal to that of the perfusion fluid. Incubation was performed at a temperature of 38 °C maintained with the aid of a heating bath and aerated with a mixture of 5 % CO_2 and 95 % O_2.

Wash-out of brain slices

After *in vivo* perfusion or *in vitro* incubation brain slices were carefully transferred by means of a forceps and quickly moved through a non-radioactive medium before being placed in a holder (Arnfred, Hertz, Lolle & Lund-Andersen, 1970). The holder was transferred through a series of test tubes containing 4 ml. non-radioactive solutions. Wash-out was performed at a temperature of 38 °C and the solutions were aerated with 5 % CO_2 and 95 % O_2. At the end of the wash-out period the final wet weight of the slices was determined with a Cahn electromagnetic microbalance (to an accuracy of ± 0·01 mg) after the slices were drained on a glass plate and adherent fluid was removed with filter paper. In addition 0·6–0·8 mm thick sheets of 1·0 % agar which had been equilibrated with ^{14}C-labelled inulin, sucrose or mannitol were washed out in the same say as the brain slices.

The radioactivity found in each test tube was added to that left in the tissue at the end of the experiment to give the total initial radioactivity of the slices. Wash-out curves were constructed as the fraction of initial radioactivity remaining in the tissue at specified wash-out times.

Radiochemicals

[^{14}C]inulin, specific activity 2·5 mc/g, [^{14}C]sucrose, specific activity 4·3 c/m-mole, [^{14}C]-D-mannitol, specific activity 50·6 mc/m-mole and [^{3}H]D-mannitol, specific activity 2·65 c/m-mole were obtained from New England Nuclear Corp. Boston, Mass., U.S.A. The purity of radiolabelled inulin, sucrose and mannitol was verified by gel filtration using a Sephadex G-25 column (Pharmacia). ^{113m}Indium was obtained from a sterile ion exchange generator.

Analyses

A Packard Tri Carb liquid scintillation spectrometer system, model 2425, was used to count tritium and ^{14}C-labelled compounds. Fifty μl. perfusate, 50 μl. incubation medium, 25 μl. plasma, 3 ml. medium and preweighed slices were placed in scintillation vials. Pieces of whole brain were weighed in the scintillation vials. Beta counting was performed after tissue solubilization. Ten ml. of scintillator solution (Instagel, Packard) was added to all the vials which were kept in darkness at 4 °C for 12 hr to eliminate chemiluminescense.

Correction of counting efficiency was performed using the external standard channel method, and standard procedure was used for simultaneous counting of ^{14}C and ^{3}H. The efficiency of counting of ^{14}C was 60 % in the ^{14}C channel and 20 % in the tritium channel, while the efficiency of counting of ^{3}H was 40 % in the tritium channel and less than 0·01 % in the ^{14}C channel. Hence, tritium could be totally excluded from the ^{14}C determinations, while one third of ^{14}C counts had to be subtracted from the tritium counts.

Gamma emission from ^{113m}Indium was counted in a well crystal counter (Packard Autogamma, model 5385) and correction for decay during the counting period was made.

Calculations

The determinations comprise measurement of total content of water and delineation of compartments in brain slices.

Total fluid content (μl./100 mg wet wt.) was determined by weighing before and after drying of the samples for 24 hr at 105 °C.

Total marker space (μl./100 mg initial wet wt.) was calculated after *in vivo* equilibration in accordance with the term

$$\frac{\text{activity per 100 mg tissue}}{\text{activity per } \mu\text{l. perfusate}}.$$

The resolution of wash-out curves was performed as described by Lund-Andersen (1974). Wash-out curves from brain slices which had been equilibrated *in vivo* could only be resolved into one exponential function and constructed on the basis of at least five observations. Each of the lines were fitted by the least-squares method. Spaces (V_T) expressed as μl./100 mg initial wet wt., were calculated in accordance with the equation

$$V_T = \frac{S}{M} \cdot \frac{100}{W} . I . 1{\cdot}6,$$

where S is the sum of radioactivity (in c.p.m.) washed out and left in the tissue, M is c.p.m. per μl. perfusion fluid, W is the final wet weight, I is the fractional intercept value on the ordinate for the slow component and 1·6 is the ratio of initial and final dry weight residue. The total marker space was corrected for intracellular penetration to obtain the extracellular distribution volume.

The amount of markers taken up by brain slices during *in vitro* incubation was expressed as spaces (μl./100 mg final wet wt.) according to the term

$$\frac{\text{activity per 100 mg tissue}}{\text{activity per } \mu\text{l. incubation medium}}.$$

Efflux curves from brain slices incubated *in vitro* were resolved into three exponentials. The resolution of desaturation curves was undertaken by peeling off technique and calculated on a Wang-600 computer to give y-intercept, half-time and ordinate value at specified time. The intercept can be expressed as space by multiplication with total marker space.

RESULTS

In vivo experiments

Tidal volume and rate of respiration was adjusted using a constant volume respirator in order to keep the arterial carbon dioxide pressure (P_{CO_2}) between 30 and 40 torr.

Arterial blood pressure averaged 95 mmHg (range 75–135 mmHg).

The concentration of [^{14}C]inulin, [^{14}C]sucrose and [^{3}H]mannitol attained in arterial blood plasma in functionally nephrectomized rats were kept within ± 10% during 5 hr perfusion. The ventriculo-cisternal perfusate/plasma water concentration ratio for the marker molecules at various times during the experiment were close to 1·00 (± 10%).

TABLE 1. [^{3}H]mannitol, [^{14}C]sucrose and [^{14}C]inulin spaces (μl./100 mg initial wet weight). In the first column the average values of the five right side cortex slices and in the second column the values from posterior part of the whole left hemisphere. Values are means ± S.D.

Compound	*n*	Right-sided slices (μl./100 mg) mean ± S.D.	Left-sided whole brain (μl./100 mg) mean ± S.D.
[^{14}C]inulin	5	14·6 ± 0·6	14·7 ± 0·9
[^{14}C]sucrose	5	18·5 ± 0·7	18·6 ± 2·1
[^{3}H]mannitol	5	27·5 ± 5·6	26·4 ± 2·6

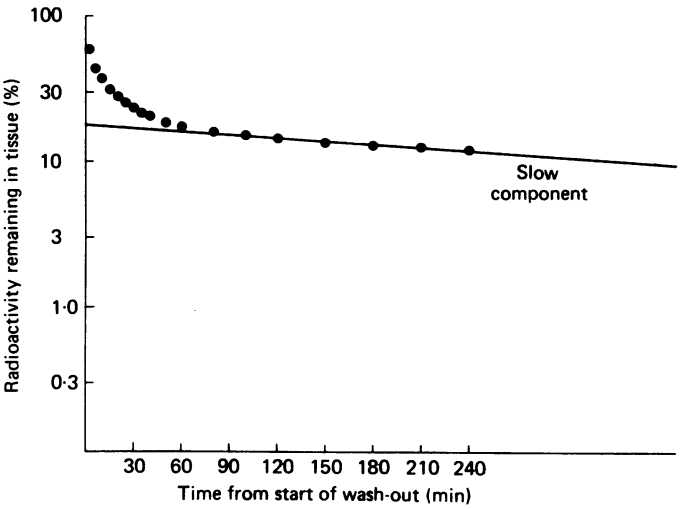

Fig. 1. Wash-out of [^{14}C]inulin from 0·6 mm thick slice of rat brain cortex exposed to the marker *in vivo* plotted in a semilogarithmic diagram. The ordinate is amount of radioactivity remaining in the slice at specified times and abscissae is time in min from start of wash-out. The straight line connecting the terminal part of the curve and extrapolated towards zero time represents the slow component. The line is fitted by eye.

Table 1 shows the distribution spaces for inulin, sucrose and mannitol in brain slices from the right part of cortex and in the posterior part of the left hemisphere following 5 hr of equilibration. It appears that mannitol distributes in part in a space not accessible to sucrose or inulin. This could be either intracellular or extracellular. In order to evaluate the possible role of intracellular penetration, wash-out from brain slices exposed to the markers *in vivo* was undertaken. Fig. 1 shows a semilogarithmic plot of the efflux of [^{14}C]inulin from 0·6 mm thick brain slice. The half-times for the slow component obtained from desaturation curves are given in Table 2 together with the amount of inulin, sucrose and mannitol located in the intracellular compartment and expressed as spaces. The calculated extracellular distribution volumes

(μl./100 mg initial wet weight) obtained by subtraction of intracellular marker space from total marker space in the slices were 13·3, 16·6 and 19·5, respectively, for [^{14}C]inulin, [^{14}C]sucrose and [^{3}H]mannitol.

TABLE 2. Half-times ($t_{\frac{1}{2}}$) for slow components, expressed as spaces for [^{14}C]inulin, [^{14}C]sucrose and [^{3}H]mannitol in 0·6 mm thick rat brain cortex slices exposed to the radiotracers *in vivo*. Values are means ± S.D.

Compound	n	$t_{\frac{1}{2}}$ for slow component (min) mean ± S.D.	Intercept value expressed as space for slow component (μl./100 mg initial wet wt.) mean ± S.D.
[^{14}C]inulin	5	363·2 ± 44·8	1·4 ± 0·2
[^{14}C]sucrose	5	384·8 ± 45·9	1·9 ± 0·4
[^{3}H]mannitol	4	187·3 ± 36·5	7·5 ± 1·4

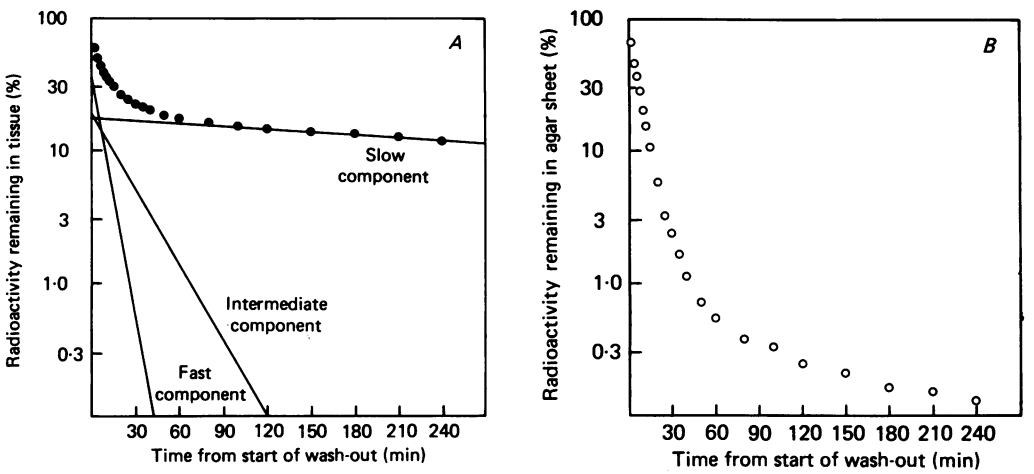

Fig. 2. *A*, wash-out of [^{14}C]inulin from 0·6 mm thick brain cortex slice following 60 min of incubation *in vitro* plotted in a semilogarithmic diagram. The ordinate is amount of radioactivity remaining in the slice at specified times and abscissae is time in min from start of wash-out. The experimental curve is shown by solid circles and resolution into three exponential functions designated slow component, intermediate component and fast component was performed as described in text. *B*, a semilogarithmic plot of efflux of [^{14}C]inulin from 0·6 to 0·8 thick agar sheet incubated for several hours with tracer inulin before wash-out.

In vitro experiments

In unincubated brain cortex slices 79·2 μl./100 mg was tissue water. After *in vitro* equilibration the difference between the total weight of swollen slice and its dry weight is the total water volume of swollen tissue. This would amount to 96·7 μl. in 109·8 mg slice. Recalculated to the basis of 100 mg slice, the amount of water volume was 88·2 μl., which means that 9 μl. fluid had been taken up in 100 mg slice during *in vitro* incubation. The difference between total fluid volume of the slice and marker space defines the tissue water not reached by the marker. The marker space would

be the fraction of tissue water in equilibrium with the marker substance in question. Following *in vitro* incubation, inulin was found to equilibrate with 32·2 μl. tissue water, sucrose with 53·8 μl. tissue water and mannitol with 66·6 μl. tissue water.

Fig. 2*A* shows a semilogarithmic plot of the time course of wash-out of [^{14}C]-inulin from brain cortex slice. The curve was resolved into three exponential functions

TABLE 3. Half-times ($t_{\frac{1}{2}}$) for slow components, intermediate components and fast components describing efflux of radiolabelled inulin, sucrose and mannitol from 0·6 mm thick rat brain cortex slices incubated in a balanced medium for 60 min with subsequent 240 min of wash-out. Values are means ± S.D.

Compound	*n*	$t_{\frac{1}{2}}$ Slow component (min) mean ± S.D.	$t_{\frac{1}{2}}$ Intermediate component (min) mean ± S.D.	$t_{\frac{1}{2}}$ Fast component (min) mean ± S.D.
[^{14}C]inulin	6	426·2 ± 83·4	15·3 ± 2·0	4·3 ± 1·0
[^{14}C]sucrose	5	239·6 ± 21·8	16·2 ± 2·0	3·4 ± 0·5
[^{14}C]mannitol	5	153·7 ± 27·5	13·5 ± 1·7	3·0 ± 0·6

TABLE 4. Magnitude of slow components, intermediate components and fast components after incubation for 60 min in a balanced medium containing radiolabelled inulin, sucrose or mannitol following 240 min of wash-out. Values are means ± S.D.

	Intercept expressed as space (μl./100 mg final wet weight)		
Compound	Slow component mean ± S.D.	Intermediate component mean ± S.D.	Fast component mean ± S.D.
[^{14}C]inulin	3·5 ± 0·3	4·2 ± 1·3	22·4 ± 1·3
[^{14}C]sucrose	7·6 ± 1·1	13·5 ± 3·3	31·7 ± 4·0
[^{14}C]mannitol	10·0 ± 1·2	13·5 ± 3·6	42·3 ± 3·5

represented by straight lines and designated slow component, intermediate component and fast component. Under the experimental conditions, the fast component serves as a measure of the extracellular space (Lund-Andersen & Hertz, 1973) and the slow component is composed of markers originated from cellular sites (Lund-Andersen & Møller, 1977). The intermediate component from which wash-out does not follow simple diffusion cannot be identified with any known anatomical compartments (Lund-Andersen, 1974). The half-times of slow component, intermediate component and fast component as obtained from the desaturation curves are given in Table 3. The magnitude of the three components obtained by multiplication of *y*-intercepts with total marker spaces are given in Table 4.

Fig. 2B shows a semilogarithmic plot of efflux of [^{14}C]inulin from 0·6 to 0·8 mm thick agar sheet incubated for several hours in tracer containing medium. Half-times (min) for efflux from agar sheets of ^{14}C-labelled inulin, sucrose and mannitol were 3·8, 1·8 and 1·2, respectively.

DISCUSSION

Uncertainty exists as to which marker gives the best estimate of the extracellular space of the brain. Studies *in vivo* are encumbered with serious problems, particularly due to the greater complexity imposed by delivery of solute indicators via cerebrospinal fluid with influence of factors such as solutes permeation into and through brain tissue fluids. The time scale required for marker equilibration with all tissue water available to it is in the order of several hours when diffusion accounts for solutes exchange (Levin & Sisson, 1972). Determination of concentration profile in brain tissue after short lasting ventriculo-cisternal or subarachnoid perfusion with the marker avoid problems concerned with true equilibrium conditions. The profile of concentration as plotted on complementary error function paper against the distance of each slice from the surface enabled Rall, Oppelt & Patlak (1962) to measure the extracellular distribution volume as the ordinate intercept. In a later study using a similar approach to examine extracellular distribution of inulin, sucrose and mannitol, Fenstermacher, Rall, Patlak & Levin (1970) have shown that the mannitol distribution volume in the dogs' brain was substantially larger than those of inulin and sucrose. This finding was confirmed in the present study where equilibrium between blood, brain extracellular fluid and cerebrospinal fluid was fully achieved or closely approached. The data presented suggest that the variability of the size of the extracellular space as determined by different marker molecules does not only depend on various fractions of the intracellular space penetrated. Some fraction of the extracellular fluid is unavailable as solvent for marker molecules due to exclusion effect at interfaces, and the fraction will increase with increasing molecular size.

The time required for equilibration of markers with tissue water during *in vitro* incubation of brain cortex slices varied with the substance used. For sucrose, inulin and protein at least 60 min incubation is required to obtain steady-state condition (Pappius, 1965). This advantage of the *in vitro* preparation is, however, countered to some extent by a pronounced swelling during incubation of the slices (Cohen *et al.* 1970). Understanding of factors concerned with swelling and the distribution within the tissue of fluid taken up is essential to the consideration of observations of solutes exchange in brain cortex slices.

In general, three types of swelling can be considered in incubated brain slices, (i) uptake of fluid by damaged tissue elements produced during tissue preparation, (ii) increase in intracellular water generated by active transport of solutes from surrounding medium or due to impaired metabolism with entry of extracellular solutes (Lund-Andersen & Hertz, 1970), and (iii) a pronounced extracellular swelling. In accordance with electronmicroscopic visualization of an extracellular space which increases during the early phase of incubation (Møller, Hertz, Mølgaard & Lund-Andersen, 1974), it has been suggested that water of swelling taken up during incubation is largely extracellular.

Measurements of extracellular distribution spaces as undertaken by the compartmental analysis of efflux from brain slices after *in vitro* equilibration has shown that spaces decrease with increasing molecular size with mannitol > sucrose > inulin. By comparison with wash-out from agar sheets and wash-out from brain cortex slices, it was shown that efflux rate from the extracellular compartment in brain slices

resembles efflux rate from agar sheets as far as inulin is concerned. This result shows that inulin is in free equilibrium with the outside medium, there being no apparent delay in the diffusion from the tissue into the medium, which indicates that the inulin space is continuous with normal extracellular fluid. In contradistinction to the

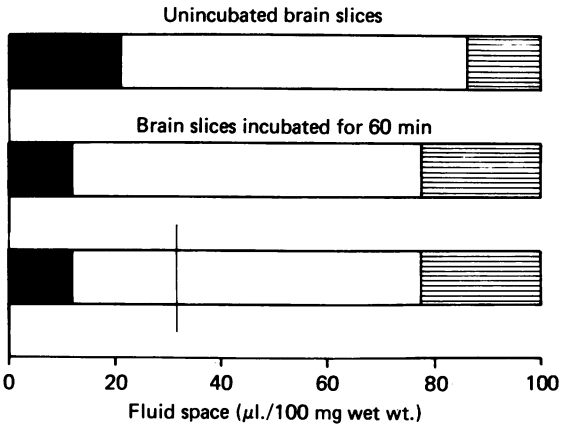

Fig. 3. Comparison of fluid spaces in slices of rat cerebral cortex following *in vivo* or *in vitro* equilibration referred to 100 mg of tissue wet weight. Black portion represents dry weight, shaded portion represents extracellular fluid space and open portion represents intracellular fluid space. In the lower bar the open portion to the right of the vertical line gives tissue water not equilibrated with inulin or mannitol, referred to intracellular fluid space of undamaged cells. For general explanation, see text.

above, efflux rates for mannitol and sucrose from brain slices were considerably slower than from agar sheets. When a slice of brain is prepared, a very large number of processes of nerve and glial cells must be cut, and the possibility exists that the large mannitol and sucrose spaces might be due to penetration of the markers into cells which have been damaged. The present results with mannitol and sucrose support the interpretation that some fraction of tissue water in equilibrium with these markers is not continuous with extracellular fluid, suggesting additional sites in a 'third space' different from extracellular and normal intracellular spaces.

The purpose of this study has been direct to correlate experiments *in vitro* with an apparently analogous situation *in vivo*. Comparison of extracellular fluid spaces *in vivo* with spaces and swelling *in vitro* shows a discrepancy in size between spaces delineated by sucrose and mannitol *in vitro* to those measured *in vivo*. Inulin space *in vitro* when allowance is made for swelling produced during incubation of the slices was similar to the chemically determined extracellular distribution space *in vivo* and, as has been shown, closely approached the morphological extracellular space (Møller *et al.* 1974).

Fluid spaces in brain cortex slices are summarized in Fig. 3. It is apparent that swelling arisen during *in vitro* incubation occurs in the extracellular space but also in a space inaccessible to inulin. This space may correspond to swollen glial cells observed in a similar material with the electronmicroscope (Møller *et al.* 1974). A part of the space inaccessible to inulin but accessible to mannitol might correspond to damaged tissue cells, whereas the space inaccessible to mannitol may represent intracellular space of grossly undamaged cells.

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