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The Osmotically Sensitive Potassium and Sodium Compartments of Synaptosomes

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1. Synaptosomes are pinched-off nerve terminals whose components can be liberated by osmotic 'shock'. A synaptosome preparation run through a Sephadex column that was eluted with an iso-osmotic solution retained its small ions, whereas when the column was eluted hypo-osmotically the small ions were lost. In this way the osmotically sensitive Na^+ and K^+ of synaptosomes were measured. Measurements of the lactate dehydrogenase occluded within the synaptosome were also made. The release of osmotically sensitive Na^+ and K^+ and occluded lactate dehydrogenase had similar characteristics with respect to the degree of osmotic 'shock' necessary and the action of lytic agents. 2. The distribution of osmotically sensitive Na^+, K^+ and occluded lactate dehydrogenase in the subfractions of a crude mitochondrial preparation was examined. The synaptosome fraction was the richest source of these constituents. 3. On standing at 5° in iso-osmotic solution Na^+ and K^+ were lost from synaptosomes, whereas the amount of occluded lactate dehydrogenase remained stable, suggesting that the synaptosome membrane retained its integrity but that Na^+ and K^+ diffused through it out of the osmotically sensitive compartment. 4. The uptake of Na^+ and K^+ into the osmotically sensitive compartment was examined. At 5° the rates of uptake of Na^+ and K^+ were found to be equal to the rates of loss of these ions when correction to a uniform concentration gradient had been made. K^+ travelled across the membrane slightly faster than Na^+, the rate of K^+ movement being about $1{\cdot}0\,\mu\mu$equiv.cm.$^{-2}$sec.$^{-1}$ under a concentration gradient of 0·1M. Active transport is not thought to contribute to the ion movements under the conditions used. 5. The amount of K^+ taken up into the osmotically sensitive compartment as a function of the external concentration was examined. Since the uncharged molecule D-[^{14}C]galactose distributes across the synaptosome membrane similarly to K^+ there is not thought to be a synaptosomal trans-membrane potential. The volume of the osmotically sensitive compartment was measured by this method and found to agree with estimates of the synaptosomal volume made from morphological studies. In media of low ionic strength synaptosomes exhibit a Donnan effect. 6. It is concluded from these studies that the osmotically sensitive compartment represents the inner volume of the synaptosome, which is completely separated from the outside environment by a diffusion barrier having many of the general properties of a biological membrane.

When mammalian cerebral cortex is homogenized in 0·32M-sucrose under carefully controlled conditions the ending of the nerve-cell axon is pinched off and may be separated as a discrete particle by density-gradient centrifugation (Gray & Whittaker, 1962; De Robertis, de Iraldi, Arnaiz & Salganicoff, 1962). Under the electron microscope such detached endings appear as membrane-bounded sacs approx. 0·5μ in diameter containing synaptic vesicles and small mitochondria, with frequently a piece of the post-synaptic membrane attached. The particles have been termed 'synaptosomes' (Whittaker, Michaelson & Kirkland, 1964). Synaptosomes contain cytoplasmic constituents such as K^+ and LDH,† and also acetylcholine, much of which is bound to the synaptic vesicles found within the synaptosome. Synaptosomes show high Na^+-plus-K^+-activated adenosine-triphosphatase activity which appears to be located on their external

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† Abbreviation: LDH, lactate dehydrogenase.

membrane (Hosie, 1965). They are osmotically sensitive, liberating most of their constituents in hypo-osmotic solutions.

The morphological and chemical evidence suggests that the synaptosome is an artificially produced sac of nerve-cell membrane containing structural and chemical components characteristic of the immediate pre-synaptic region. The fact that soluble cytoplasmic constituents are retained within the membrane during a 4hr. preparative procedure suggests that even if the membrane is not fully functional it has sealed sufficiently to prevent the escape of the soluble components. The compartment segregated by the synaptosome membrane makes it possible to examine processes taking place across the membrane. In this study methods for the investigation of the osmotically sensitive compartment of synaptosomes have been examined, and some characteristics of the osmotically sensitive K^+, Na^+ and LDH have been established. In this way it is hoped to develop methods for the study of the processes taking place across the synaptosome membrane. An account of this work was presented at the July 1966 meeting of The Biochemical Society (Marchbanks, 1966).

METHODS

Preparation of synaptosomes

Isolation from tissue. Synaptosomes were prepared from guinea-pig or coypu (*Myocastor coypus*) cortex from which the white matter had been removed before homogenization. The synaptosomes from the two species were found to be identical in behaviour in the experiments performed. The isolation procedure used was as described by Gray & Whittaker (1962) for fraction *B*, except that to minimize microsomal contamination the crude mitochondrial fraction (P_2) was usually sedimented at 10000*g* for 20min. Fractions *A* (myelin) and *C* (mitochondria) prepared at the same time were discarded unless otherwise stated. The difference of diameter between the pestle and mortar of the homogenizer was 0·025cm. and the speed of rotation of the pestle was 840rev./min. Sucrose solutions were brought to pH7·4 at 5° with tris–HCl. Unless otherwise reported, preparations of synaptosomes were kept as isolated in 0·8M-sucrose at 5° and used within 36hr. of preparation. The amount of a pellet or suspension derived from 1g. (wet wt.) of cortex is referred to as 1g.-equiv. of fraction. Normally concentrations of 0·1–0·2g.-equiv. of fraction/ml. were used.

Ultrasonic disruption. Synaptosome preparations were submitted to ultrasonic vibrations at 20000cyc./sec. from a 60w Mullard–MSE disintegrator (type 7685/2 with a titanium probe) for various times at 2°.

Analytical methods

Determination of K^+ *and* Na^+. K^+ and Na^+ were determined on an EEL model A flame photometer. Neither protein, nor sucrose nor Na^+ in the concentrations usually present interfered with the estimation of K^+. Sucrose and K^+ interfered slightly (5%) with the estimation of Na^+ and accordingly special calibration curves were used when Na^+ was estimated in the presence of these substances. Results are expressed as μg.atoms/g.-equiv.

Assay of lactate dehydrogenase. This was carried out as described by Johnson (1960). The change in E_{340} that accompanies the transfer of hydrogen from $NADH_2$ to sodium pyruvate at 22° was measured in a Unicam SP.700 recording spectrophotometer.

Measurement of radioactivity. The radioactivity of D-[^{14}C]galactose and $^{36}Cl^-$ was measured in a Packard automatic scintillation counter by using the scintillation medium described by Bray (1960). Changes in relative counting efficiency due to quenching were always checked and corrected for when scintillation counting was used. The radioactivity of ^{131}I-labelled human serum albumin was determined with a thallium-activated NaI-crystal detector attached to a Panax T300/D657 time-scaler. It was established that 99·9% of the radioactivity of the ^{131}I-labelled human serum albumin appeared in the void volume of a Sephadex column. No attempt was made to measure the specific activity of these radioactive substances, since I was only concerned with the equilibration of the radioactivity. All the radioactive substances were purchased from The Radiochemical Centre, Amersham, Bucks.

Determination of protein. This was carried out by the method of Lowry, Rosebrough, Farr & Randall (1951), with crystalline bovine plasma albumin as standard.

Determination of osmotically sensitive K^+ *and* Na^+. Pairs of small columns (11cm. high × 0·8cm. diam.) containing 0·5g. of Sephadex G-50 (bead form) were used. One column (the iso-osmotic column) was eluted with a buffered sucrose solution iso-osmotic to that in which the synaptosomes were suspended, i.e. 0·8M. Another column (the hypo-osmotic column) was eluted with 5mM-tris–HCl buffer, pH7·4. A sample (0·5ml.) of the synaptosome preparation was added to the top of each column and allowed to drain down; 0·8ml. of eluting solution of the appropriate osmolarity was then added and also allowed to drain down; then a further 1·0ml. was added and the effluent collected, the whole procedure taking 3–5min. The recovery of synaptosomal protein in the effluent was 80% and that of unbound small molecules less than 1·0%. The columns were then washed with 50ml. of eluting solution and used again. The performance of the columns is discussed in more detail in the Results section.

The K^+ or Na^+ in the effluent of the column eluted with iso-osomotic solutions is principally that contained within the synaptosome plus that bound to free membranes and protein. The amount of Na^+ and K^+ in the effluent of the hypo-osmotic column is very much less, the synaptosomes having become hypo-osmotically 'shocked' and thus having lost their internal small ions during their passage through the column. There is a small amount of K^+ and Na^+ in the effluent from the hypo-osmotic column, presumably mainly representing that bound to protein and free membranes in the preparation. The columns were run at room temperature, it having been established that no advantage in recovery of osmotically sensitive K^+ was to be gained by running the columns at 5°. The difference between the amount of small ion present in the effluent from the column eluted iso-osmotically and that eluted hypo-osmotically is termed the osmotically sensitive small-ion content of the synaptosome preparation. It is denoted by the subscripts

(K_s^+, Na_s^+) and expressed in μg.atoms/g.-equiv. of fraction. Estimates of K_s^+ and Na_s^+ had a 10% standard deviation by this method.

Determination of occluded lactate dehydrogenase. Synaptosome preparations treated with 1% (w/v) (final concn.) Triton X-100 show a greatly increased LDH activity because the synaptosome membrane is destroyed by the detergent and the LDH occluded within the synaptosome rendered accessible to its cofactor and substrate. The ratio of LDH activity with and without Triton has been used as a measure of the ratio of free to total LDH. All the LDH can be converted into the free form by osmotic 'shock' (see the Results section). The ratio of free to total LDH was determined as follows. A sample of synaptosome preparation was added to the cuvette containing the reagents, in the spectrophotometer, stirred and the change in E_{340} with time recorded for about 1 min. Then 0·2 ml. of 10% (w/v) Triton X-100 was added to the cuvette, the mixture was stirred and the change in E_{340} was recorded for a further 1 min. The rate of change of E_{340} with time before the addition of Triton represents the activity of free LDH, and that after the addition of Triton the activity of the total LDH, of the synaptosome preparation. The ratio of free to total activity can be found from the ratio of the slopes on the chart record. The occluded LDH was not released into reaction mixture during the 1–2 min. necessary to measure the activity of free LDH, since no increase in rate could be observed over this period. Therefore occluded LDH does not contribute to the activity found before the addition of Triton, and so the ratio of occluded to total LDH is 1 − free LDH/total LDH. The action of Triton in releasing occluded LDH was found to be instantaneous. The standard deviation of estimates of the ratio of occluded to total LDH was 12% by this method.

RESULTS

Determination of the amounts of osmotically sensitive components. Fig. 1 shows the performance of the Sephadex columns with respect to K^+ and protein. Fig. 1(*a*) shows that when a synaptosome preparation was run through a column eluted with 0·8 M-sucrose there were two peaks of K^+ concentration, the first associated with the protein peak. When the same preparation was run through a column eluted hypo-osmotically (Fig. 1*b*) only one peak of K^+ concentration was observed, that associated with the protein peak having been lost. During hypo-osmotic 'shock' the synaptosome membrane evidently became more permeable to K^+, which leaked out and was retained in the interior volume of the Sephadex column. In Table 1 a comparison of methods of releasing K^+ and occluded LDH of synaptosomes is given. Whereas the Sephadex columns separate free ions from bound ions, no separation of a macromolecule like LDH occurs on these columns. The K^+ and occluded LDH as a percentage of the total were approximately the same after passing through the iso-osmotic column. After passing through the hypo-osmotic column most of the K^+ is lost and there is no

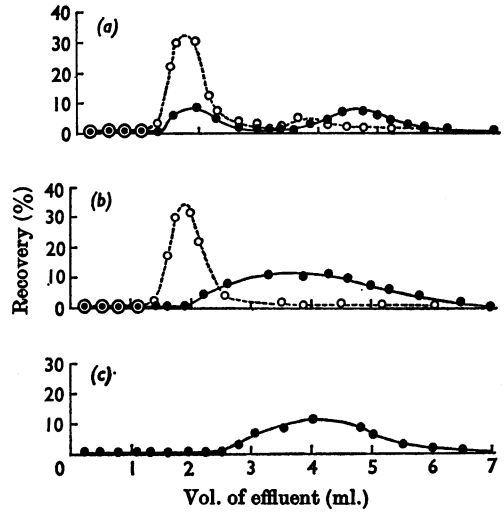

Fig. 1. Performance of Sephadex columns. ●, K^+; ○, protein. (*a*) 0·5 ml. of synaptosome preparation eluted with 0·8 M-sucrose; (*b*) 0·5 ml. of synaptosome preparation eluted with 5 mM-tris–HCl buffer, pH 7·4; (*c*) 0·5 ml. of 2·5 mM-KCl eluted with 0·8 M-sucrose.

Table 1. *Comparison of methods for releasing* K^+ *and occluded lactate dehydrogenase from a freshly isolated synaptosome preparation*

For details of methods see the text. The amount of osmotically sensitive constituent is the difference between the amounts separated under iso-osmotic and hypo-osmotic conditions.

Treatment	K^+ (% of total)	Occluded LDH (% of total)
Iso-osmotic column (0·8 M-sucrose)	35	42
Hypo-osmotic column (5 mM-tris)	2	0
Pretreatment with 1% Triton X-100, then:		
Iso-osmotic column	1	0
Hypo-osmotic column	0·5	2
Ultrasonic treatment for 30 sec., then:		
Iso-osmotic column	8	10
Hypo-osmotic column	0	0
Present in rinsed pellet after centrifugation for 30 min. at 12000*g* in:		
0·4 M-Sucrose	35	48
0·02 M-Sucrose	4	18
Present in rinsed pellet after centrifugation for 60 min. at 110000*g* in:		
0·4 M-Sucrose	48	—
0·02 M-Sucrose	2	—

occluded LDH. The columns therefore have the same effect on osmotically sensitive K^+ and occluded LDH, except that free K^+ is separated from bound K^+ by the column whereas free and occluded LDH are not.

When a synaptosome preparation was treated with 1% (w/v) Triton X-100 and then run through the columns very little K^+ or occluded LDH could be detected in the void volume effluent of either the iso-osmotic columns or the hypo-osmotic columns. This indicated that treatment with Triton liberates osmotically sensitive K^+, presumably in the same way that it renders occluded LDH accessible to its substrate and cofactors. Ultrasonic treatment also released K^+ and occluded LDH. The osmotically sensitive K^+ was also separated by centrifuging, rinsing the pellets without resuspension and determining the K^+ in the pellets. When the centrifuging was carried out in a hypo-osmotic medium the pellet contained very little K^+. Comparison of the K^+ contents of pellets spun down at different speeds showed that 75% of the osmotically sensitive K^+ is sedimented at 12000*g* for 30min. More occluded LDH appeared in pellets sedimented from 0·4M-sucrose than in those sedimented from 0·02M-sucrose, which correlates with the results for K^+; however, the proportion of occluded LDH in the hypo-osmotic pellet was much higher. This could be due to incomplete separation of the enzyme from the hypo-osmotically 'shocked' synaptosome.

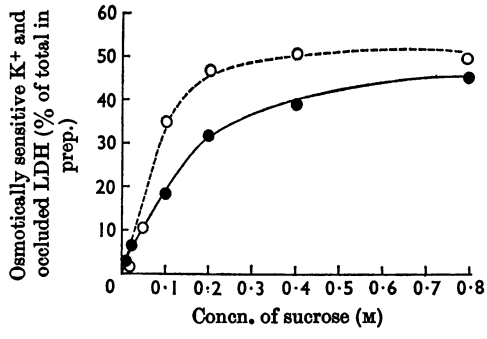

Fig. 2. Dependency of K^+ loss and LDH occlusion on degree of osmotic 'shock'. ●, K^+; ○, occluded LDH.

The dependency of K^+ loss from the osmotically sensitive compartment and LDH occlusion on the degree of osmotic 'shock' was investigated by eluting the columns with sucrose solutions of intermediate molarity. The results (Fig. 2) show that osmotically sensitive K^+ and occluded LDH behave similarly with respect to the degree of osmotic 'shock'. Both are moderately stable at concentrations of sucrose above 0·4M, but free K^+ and LDH appear rapidly as the concentration is decreased below 0·4M.

Distribution of osmotically sensitive components. The distributions of osmotically sensitive K^+ and Na^+ and of occluded LDH were examined in subfractions of a crude mitochondrial fraction. The percentage distributions and relative specific activities with respect to protein are shown in Table 2. There is more osmotically sensitive K^+ in the synaptosome fraction than in either fraction *A* (myelin) or fraction *C* (mitochondria). A similar pattern is seen with the distribution of osmotically sensitive Na^+ and occluded LDH. The relative specific concentrations with respect to protein of osmotically sensitive K^+ and Na^+ and of occluded LDH are highest in the synaptosome fraction (*B*), indicating that this subfraction is the most concentrated source of osmotically sensitive components.

Stability of osmotically sensitive components. The stability of the osmotically sensitive components was examined by investigating the changes on

Table 2. *Distribution of osmotically sensitive* K^+ *and of occluded lactate dehydrogenase in subfractions of a crude mitochondrial fraction*

For preparation and analysis of fractions see the text. The variability recorded is the S.E.M. (four determinations).

	Fraction *A*	Fraction *B*	Fraction *C*
Centrifugal characteristics	Lighter than 0·8M-sucrose	Density between 0·8M- and 1·2M-sucrose	Density greater than 1·2M-sucrose
Morphology	Myelin and membrane fragments	Synaptosomes	Mitochondria
% of total protein recovered in fraction	30±5	54±5	16±4
% of total recovered osmotically sensitive K^+	20±4	75±5	5±1
Relative specific concentration	0·66	1·4	0·31
% of total recovered osmotically sensitive Na^+	13±18	76±13	11±7
Relative specific concentration	0·44	1·4	0·69
% of total recovered occluded LDH	28±4	63±3	9±1
Relative specific concentration	0·93	1·17	0·55

standing at 5° in 0·8M-sucrose. The results are plotted in Fig. 3. A sharp difference was observed between the behaviour of osmotically sensitive K^+ and Na^+ on the one hand and occluded LDH on the other. Whereas the ratio of occluded to total LDH remained stable for up to 40hr., osmotically sensitive K^+ and Na^+ were lost rapidly.

The effect of glucose and ATP with appropriate concentrations of Mg^{2+} and Ca^{2+} on the K^+ loss at 5° was examined. Neither the rate of loss nor the final equilibrium position were effected by the addition of these energy-supplying substrates. The addition of ouabain (final concn. 100μM) had no effect either in the presence of or without glucose or ATP.

Although no changes in the amount of occluded LDH were observed at 5°, a loss of occluded LDH was observed when the experiment was repeated at a higher temperature. It was also observed that the rate of loss of osmotically sensitive K^+ increased, and the final equilibrium amount was lower than at 5° (Fig. 4).

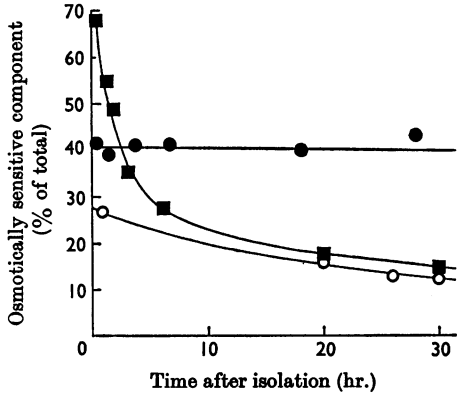

Fig. 3. Stability of osmotically sensitive components. ■, K^+; ○, Na^+; ●, occluded LDH.

Uptake into the osmotically sensitive volume. The uptake of K^+, Na^+ and the radioactivity of D-[^{14}C]-galactose into the osmotically sensitive compartment was studied by adding these substances to the synaptosome preparation in 0·8M-sucrose. The final concentration of K^+ and of Na^+ was 100mM. Fig. 5 shows the fractional approach to equilibrium of K^+ as a function of time (mean of two experiments).

The fractional rate is dF/dt (sec.$^{-1}$), where F is defined as $(X_t - X_0)/(X_e - X_0)$, X_t is the amount of component X in the osmotically sensitive compartment at time t, and X_0 and X_e are the amounts at zero time and at equilibrium respectively. The initial fractional rates were taken as the rate measured during the first 20min. after additions. To convert these values into a more readily comparable rate per unit area of membrane ($\mu\mu$moles cm.$^{-2}$sec.$^{-1}$) the initial fractional rate is multiplied by $(X_e - X_0)$ for that experiment to give the rate of entry (moles sec.$^{-1}$) into 1g.-equiv. of synaptosomes. The surface area of the osmotically sensitive compartment was computed by assuming that it is the same as the surface area of the synaptosome. A value of $0{\cdot}285 \times 10^{-4}$cm. was taken for the mean synaptosome radius (Clementi, Whittaker & Sheridan, 1966). From the same paper it is possible to compute a total volume of the synaptosomes in 1g.-equiv. of fraction given the distribution of the diameter and the number of synaptosomes/g.-equiv.; this was found to be 31·5μl. for 1g.-equiv.

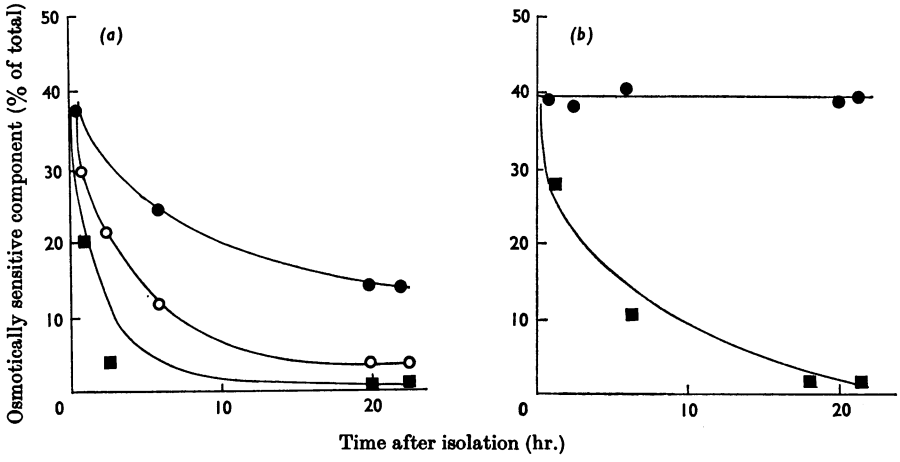

Fig. 4. Stability of osmotically sensitive components at different temperatures. (*a*) K^+ at 5° (●), at 23° (○) and at 37° (■); (*b*) occluded LDH at 5° (●) and at 37° (■).

containing $1{\cdot}79 \times 10^{11}$ synaptosomes. The surface area of 1 g.-equiv. of synaptosomes is $1{\cdot}82 \times 10^3$ cm.^{2}. In experiments on the rate of uptake, the concentration gradient was under experimental control since the synaptosomes were allowed to lose their freely diffusible osmotically sensitive components by standing for 24 hr. before the uptake experiment. In the experiments on the rate of loss of endogenous osmotically sensitive K^+ and Na^+ the concentration gradient depended on the amount of these substances within the osmotically sensitive compartment at the time of isolation. This amount $(X_0 - X_e)$ was converted into a concentration by dividing by the morphological estimate of volume, i.e. 31·5 μl/g.-equiv. The uptake and loss rates so calculated are recorded in Table 3, having been corrected to the value for a concentration gradient of 0·1 M assuming proportionality of the rates with concentration gradient within the range of concentration gradients found (0·04–0·17 M).

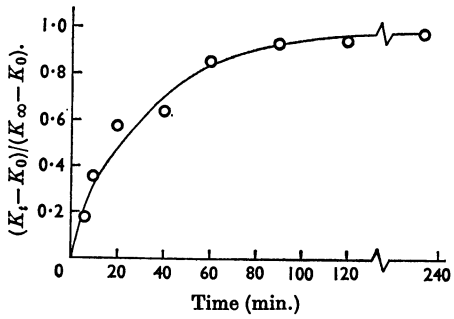

Fig. 5. Fractional approach to equilibrium of K^+ entering the osmotically sensitive compartment. K_t is the osmotically sensitive K^+ at time t, K_0 is that at time zero and K_∞ is that at equilibrium (more than 3 hr.). The concentration of K^+ was 100 mM.

It is thought that the synaptosome trans-membrane potential is zero or negligible under the conditions used (see next section), in which case the chemical potential gradients of Na^+ and K^+ are equal to their electrochemical potential gradients. If passive diffusion is the only process taking place across the membrane, rate of loss should equal rate of uptake under conditions of equal electrochemical potential gradients (Ussing, 1952). The rate of loss is approximately equal to the rate of uptake under equal concentration gradients for both Na^+ and K^+, thus suggesting that the ion movements are due to passive diffusion, assuming the equivalence of gradients of concentration and chemical potential.

The variability in rate values is almost certainly due to variabilities in the osmotically sensitive volume between different preparations. If no processes other than passive diffusion take place across the membrane the K^+ equilibrium volume (see the next section) may be used as the estimate of volume of the osmotically sensitive compartment in any particular preparation. The rate values calculated in this manner (Table 3) are approximately the same but less variable than those calculated from the single volume estimate. The concentration immediately after isolation of endogenous osmotically sensitive K^+ was 0·088 M (S.E.M. ±0·025 M; five determinations) and that of Na^+ was 0·054 M (two determinations).

Given that diffusion is the only process across the membrane and that the membrane potential is zero, the permeability constant (in cm.sec.$^{-1}$) may be computed from the rates by dividing them by the concentration difference in moles cm.$^{-3}$. The

Table 3. *Diffusion of* K^+ *and* Na^+ *into and out of the osmotically sensitive compartment in synaptosome preparations*

For measurement of initial fractional rate of diffusion see the text.

	K^+ loss	K^+ uptake	Na^+ loss	Na^+ uptake	K^+ uptake	Radioactivity (as D-[^{14}C]-galactose) uptake
No. of expts. with different preparations	3	2	2	2	2	2
Ionic strength (M)	0·2	0·2	0·2	0·2	0·1	0·1
Diffusion as measured by:						
$10^3 \times$ Initial fractional rate (sec.$^{-1}$)	0·91–1·01	1·5–1·9	0·34–0·56	1·3–1·6	1·3–1·4	2·0–2·1
Rate ($\mu\mu$moles sec.$^{-1}$ cm.$^{-2}$) under concn. gradient of 0·1 M, from morphological estimate of synaptosome volume	1·40–1·51	0·91–1·52	0·93–0·95	0·64–1·10	0·88–1·19	
Rate (as above), but from K^+ equilibrium volume of each preparation	0·88–1·01	0·96–1·21	0·34–0·54	0·66–0·90	0·72–0·78	
$10^9 \times$ Permeability constant (cm. sec.$^{-1}$)	10·1		6·1		7·5	26

permeability constants in Table 3 were obtained from the midpoints of the combined means of the rates calculated by using the K^+ equilibrium volume.

Amount taken up into the osmotically sensitive compartment. The amount taken up into the osmotically sensitive compartment at equilibrium was investigated as a function of the external concentration. The points of Fig. 6 show the amount of K^+ that is taken up into the osmotically sensitive compartment as a function of the external K^+ concentration; the sucrose concentration was 0·8 M throughout.

If the osmotically sensitive compartment is regarded as a volume separated from the external medium by a semi-permeable membrane across which no forces other than passive diffusion operate, and across which there is no membrane potential, the volume may be calculated by dividing the amount of osmotically sensitive substances at equilibrium by its external concentration, as given by:

$$X_s = V_s[X_0] \quad (1)$$

where X_s is the amount of osmotically sensitive X, V_s is the osmotically sensitive volume and $[X_0]$ is the external concentration of X. The average volume of six preparations so measured at an external K^+ concentration of 100 mM was 23·8 ± 4·3 μl./g.-equiv. of synaptosomes. Table 4 presents the ratio $X_s/[X_0]$ for Na^+, and for radioactivity as D-[^{14}C]galactose and ^{131}I-labelled human serum albumin, compared with the K^+ value for that preparation. The values are presented relative to the K^+ value for any given preparation because there was some variability in K^+ values between different preparations (S.D. ± 71% of mean; eight determinations). Table 4 shows that the volume of the K^+ compartment is the same as those of the Na^+ compartment and the D-[^{14}C]galactose compartment. D-Galactose, being an uncharged molecule, will distribute according to its concentration gradient. K^+, being a charged ion, distributes according to its electrochemical potential, which is equal to its chemical potential only if the synaptosome trans-membrane potential is zero. Since the distribution across the synaptosomal membrane is the same for both D-galactose and K^+, the synaptosome trans-membrane potential is thought to be negligible and eqn. (1) to be valid for charged ions under these experimental conditions. The very low value for ^{131}I-labelled human serum albumin indicates that the osmotically sensitive compartment is inaccessible to macromolecules.

An attempt to measure the rate of uptake and osmotically sensitive volume of $^{36}Cl^-$ gave anomalous results. $K^+{}^{36}Cl^-$ (100 mM) was added to the synaptosome preparation and the uptake of $K^+{}^{36}Cl^-$ into the osmotically sensitive compartment measured. At equilibrium with respect to K^+ the amount (in moles) of $^{36}Cl^-$ in the osmotically sensitive compartment was much less than that of K^+. This could be because the permeability of the synaptosome membrane to $^{36}Cl^-$ is much greater than that of K^+, so that when the synaptosomes loaded with $^{36}Cl^-$ are passed through the Sephadex column $^{36}Cl^-$ is lost rapidly by exchange with the chloride in the eluting solution. When this type of experiment was repeated in 0·4 M-tris–hydrochloric acid buffer, which because of its low viscosity permits the columns to be run much faster (30 sec. as opposed to 3–5 min. for 0·8 M-sucrose), the $^{36}Cl^-$ volume was found to be much closer to, though still less than, the K^+ volume. The low $^{36}Cl^-$ volume found when experiments were done in sucrose

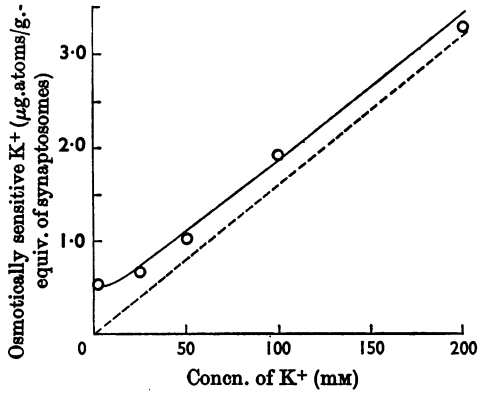

Fig. 6. Osmotically sensitive K^+ as a function of concentration of K^+. The points are experimental results. The unbroken line is the theoretical curve of eqn. (3), obtained by using $V_s = 16\,\mu$l./g.-equiv. of synaptosomes and $[Z_s^-] = 0{\cdot}5\,\mu$equiv. of non-diffusible anion/g.-equiv. of synaptosomes. The broken line is the theoretical curve for eqn. (3) obtained by using $V_s = 16\,\mu$l./g.-equiv. of synaptosomes and $Z_s^- = 0$.

Table 4. *Volume of the osmotically sensitive compartment* ($X_s/[X_0]$) *relative to the volume for* K^+ *for that preparation*

The variability recorded in the S.E.M. (three determinations, performed on different preparations of synaptosomes).

Substance	Volume relative to K^+ volume
Na^+	1·07 ± 0·18
Radioactivity (as D-[^{14}C]galactose)	0·99 ± 0·22
Radioactivity (as ^{131}I-labelled human serum albumin*)	0·05 ± 0·04

* Determined by centrifugation method.

would then be explicable as re-equilibration losses from the osmotically sensitive compartment as it passes down the Sephadex column.

Fig. 6 shows that the amount of K^+ in the osmotically sensitive compartment does not tend to zero as the external K^+ concentration approaches zero, as would be expected if eqn. (1) were followed. It was also noted that synaptosomes allowed to lose K^+ from the osmotically sensitive compartment retained more K^+ than could be accounted for by the K^+ volume measured at 100 mM external K^+ concentration. This effect could be caused by the presence of a non-diffusible anion inside the osmotically sensitive compartment (i.e. intrasynaptosomal protein) exerting a Donnan effect. To substantiate this hypothesis and estimate the magnitude of this effect, the following experiment was done. It follows from the theory of Donnan equilibria (Harris, 1956, p. 62) that, if a second diffusible cation (Na^+) with common anion (Cl^-) is added, the Donnan disequilibrium with respect to K^+ will be depressed. The fundamental equations for the distribution of charge and activity become:

$$\left.\begin{aligned}[Cl_o^-] &= [Na_o^+]+[K_o^+]\\ [Z_s^-]+[Cl_s^-] &= [Na_s^+]+[K_s^+]\end{aligned}\right\}$$ by condition of electroneutrality on each side of the membrane

$$\left.\begin{aligned}[Na_o^+][Cl_o^-] &= [Na_s^+][Cl_s^-]\\ [K_o^+][Cl_o^-] &= [K_s^+][Cl_s^-]\end{aligned}\right\}$$ by condition of equality of electrochemical activity on each side

where $[Na_o^+]$ etc. represent the concentrations of Na^+ etc. in the external medium, and $[Na_s^+]$ etc. refers to concentrations in the osmotically sensitive compartment. $[Z_s^-]$ is the concentration of non-diffusible anion in equiv./l. volume of the osmotically sensitive compartment. By substitution and introduction of eqn. (1) for the species $[K_s^+]$ and $[Z_s^-]$ the following expression is obtained:

$$(K_s^+)^2/[K_o^+] = Z_s^-K_s^+/([Na_o^+]+[K_o^+]) + V_s^2[K_o^+] \quad (2)$$

Therefore a plot of $(K_s^+)^2/[K_o^+]$ against $K_s^+/([Na_o^+]+[K_o^+])$ is linear and has a slope of Z_s^-. Such a plot is shown in Fig. 7 and the value of Z_s^- for this preparation is 1·3 μequiv. of non-diffusible anion/g.-equiv. of synaptosomes.

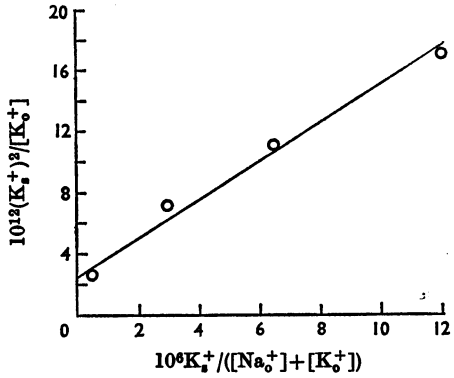

Fig. 7. Effect of NaCl on osmotically sensitive K^+ at low K^+ concentration (4 mM). K_s^+ is in g.atoms/g.-equiv. of synaptosomes, $[Na_o^+]$ and $[K_o^+]$ are molar concentrations. The slope equals equiv. of non-diffusible anion/g.-equiv. of synaptosomes $[Z_s^-]$ (see eqn. 2).

Values were found to vary between 0·4 and 2·0 μ-equiv. of non-diffusible anion/g.-equiv. for different synaptosome preparations. Having obtained a value for Z_s^- it is possible to construct a theoretical curve of K_s^+ as a function of $[K_o^+]$ to compare with the experimental points of Fig. 6. The appropriate expression, derived from the fundamental equations describing the Donnan equilibrium and solved for K_s^+, is:

$$K_s^+ = \{Z_s^- \pm ((Z_s^-)^2+4V_s^2[K_o^+]^2)^{\frac{1}{2}}\}/2 \quad (3)$$

The value of Z_s^- for this preparation was found to be 0·5 μequiv./g.-equiv. Taking V_s to be 16 μl./g.-equiv. of synaptosomes because at maximum $[K_o^+]$ the osmotically sensitive volume tends to this value, the theoretical curve of Fig. 6 was drawn. There is a reasonable correlation between the experimental points and the theoretical curve. Taking average values of V_s and Z_s^- the volume estimates obtained by using an external K^+ concentration of 100 mM will be 6% in error.

The volume of a synaptosome pellet was measured by centrifuging a synaptosome preparation in 0·4 M-sucrose at 110000 *g* for 1 hr. in a thin tube of known diameter. The volume of the pellet, minus the dead space measured with ^{131}I-labelled human serum albumin, varied between 50 and 70 μl./g.-equiv. of synaptosomes. The dead space of the pellets was over 50% of the total volume. The effect of different osmolarities in the external medium on the K^+ volume was examined by exposing the synaptosome preparation to different concentrations of sucrose along with 100 mM-K^+. The K^+ volume was found to be invariant over the range of external concentration 0·4–1·2 M when 12 hr. was allowed for equilibration.

DISCUSSION

As a prerequisite to the study of the pre-synaptic membrane and the processes that take place across it as exemplified in synaptosomes, it is necessary to establish methods for handling them and for measuring the constituents inside them. The experiments described were undertaken to establish methods for the investigation of the magnitude and

properties of the interior compartment of synaptosomes. It must be clear that the methods used are actually measuring material inside the sealed synaptosome, and not just material bound to the fragmented membranes and other cell debris present in the preparation. The criterion adopted in these studies has been that of osmotic sensitivity because it provided a guarantee that constituents so studied are actually inside the synaptosome and not merely bound to its membrane.

The increase in permeability of the erythrocyte on hypo-osmotic 'shock' (osmotic haemolysis) is well known (see review by Ponder, 1952). The release of osmotically sensitive components by ultrasonic treatment has a parallel in the haemolysis of erythrocytes by ultrasonic treatment (Guillet & Fowler, 1954). The disruption of synaptosome membranes by ultrasonic treatment has been observed in the electron microscope (Maynert, Levi & Lorenzo, 1964). A further similarity between the synaptosome and the erythrocyte is the susceptibility of the containing membrane to lysis by detergents (Pethica & Schulman, 1953). These similarities between the behaviour of the synaptosome and the erythrocyte support the view that the osmotically sensitive components are enclosed by a membrane having the general properties of biological membranes as exemplified by the erythrocyte. The similarity of the effects of osmotic 'shock', detergents and ultrasonic treatment on the occluded LDH and osmotically sensitive K^+ suggests that one is in fact measuring the accessibility of occluded LDH to its substrate and cofactor and therefore the permeability of the occluding membrane to these substances.

The distribution and relative specific activity of osmotically sensitive components in the mitochondrial subfractions suggests that the phenomena of osmotic sensitivity is specifically associated with synaptosomes. The gradual loss of osmotically sensitive K^+ and Na^+ on standing is due to passive diffusion down their concentration gradients. The failure of glucose or ATP to influence the rate of loss or final equilibrium position indicates that energy-requiring processes do not contribute to the ion movements out of the osmotically sensitive compartment under the conditions used. The constancy of the amounts of occluded LDH for up to 40 hr. on standing at 5° suggests that the synaptosome retains its structural integrity and does not open or rupture under these conditions. However, at higher temperature occluded LDH is lost, suggesting that at 37° (and to a smaller extent at 23°) synaptosomes are not stable and tend to disintegrate. Loss of structure of synaptosomes at elevated temperatures has been observed in the electron microscope (Whittaker, 1962).

The studies of the rate of diffusion of K^+ and other substances into the osmotically sensitive compartment are strictly preliminary in nature. No attempt has been made to supply a more physiological environment than 0·8 M-sucrose. Krebs, Eggleston & Terner (1951) found that the turnover rate of K^+ exchange in guinea-pig brain slices was 4%/min.; the rate in synaptosomes calculated from the initial rate of entry and synaptosomal volume is 4·5%/min. The rate of movement of K^+ across the synaptosome membrane is comparable with the value found for resting efflux and influx of K^+ in mammalian non-myelinated nerve fibres (Keynes & Ritchie, 1965). The permeability constant of the synaptosome membrane to K^+ was found to be rather larger than that of the erythrocyte. It is noteworthy that the ratio of the permeability constants of the membrane to K^+ and Na^+, namely 1:0·605, is roughly equal to the ratio of their ionic mobilities, namely 1:0·681 (Glasstone & Lewis, 1940).

The relation of the K^+ equilibrium volume per g.-equiv. of synaptosomes (23·8 μl.) to the volume obtained from the morphological study by Clementi *et al.* (1966) is as follows. Correcting the K^+ equilibrium volume for the column recovery factor (80%) and overestimation due to the Donnan equilibrium (6%), the value obtained is 28·0 μl./g.-equiv. It has been estimated that 24% of the intra-synaptosomal volume is taken up by mitochondria (V. P. Whittaker, personal communication). My observations on the distribution of osmotically sensitive components indicate that mitochondria isolated from the density gradient do not have an osmotically sensitive compartment that is measurable by the methods used and that therefore the osmotically sensitive volume underestimates the intra-synaptosomal volume by 24%. Applying this correction, the value comparable with the morphologically estimated volume of 31·5 μl. would be 38 μl./g.-equiv. of synaptosomes. The difference between the two estimates is considered reasonable, especially when it is appreciated that the process of fixation for electron-microscope examination may have caused some shrinkage. The pellet volume inaccessible to ^{131}I-labelled serum albumin is higher than both these estimates of the synaptosome volume. This seems likely to be due to the presence in the preparation of contaminating particles that do not have an osmotically sensitive compartment.

The existence of a Donnan effect in environments of low ionic strength is consistent with the impermeability of the membrane to protein. Synaptosomes are thought to be formed during the initial homogenization by a pinching-off process from the nerve-cell axon. It seems likely that small variations in preparative procedure at this stage might have a disproportionate effect on the yield of synaptosomes in good condition, which might

account for the variability of the K^+ equilibrium volume between different preparations. The invariance of the osmotically sensitive volume with the osmolarity of the environment is at first sight surprising, since by analogy with the erythrocyte the synaptosome might be expected to behave as an osmometer. However, the volume change would only be measured in my experiments if it persisted longer than 12hr. A transitory and reversible volume change would not be detected.

It is my conclusion from these studies that the osmotically sensitive compartment represents the inner volume of the synaptosome. It is completely separated from the outside environment by a membrane with many of the general properties of a biological membrane. In particular the turnover rates across the membrane suggest that it is substantially intact, although I have not established that the membrane is fully functional in the sense that active transport takes place across it. The synaptosome preparation is thus a readily available preparation of sealed nerve-cell membrane, and as such could be used for the investigation of the properties of the nerve-cell membrane *in vitro*. Such a preparation from the central nervous system has not hitherto been available. The synaptosome preparation has a further claim to uniqueness in that it is predominantly pre-synaptic membrane, and is therefore worthy of investigation because of the involvement of the pre-synaptic region in the processes transmitting impulses from one nerve cell to the next.

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