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Passive Ionic Permeabilities of Synaptosomal Membranes from Rat Brains*

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Summary. Passive ionic permeabilities of synaptosomal membranes from rat brains were measured under various ionic conditions of intra- and extrasynaptosomal solutions by means of radioisotopic tracers, and were treated quantitatively. The efflux rates of Na^+ and K^+ ions were observed to be affected by the concentrations of permeable monovalent cations inside and outside the membranes, and not by other factors such as the osmotic pressure or the concentrations of anions. The rates increased with the outer concentrations of permeable monovalent cations, and decreased with the inner ones. Na-efflux was most efficiently accelerated by Na^+ ions in the outer solution, and K-efflux by K^+ ions. At the optimal conditions, K^+ ions traversed the membranes much faster than Na^+ ions. Cl-efflux was observed to be independent of the ionic condition of the outer solution.

These observations could not be explained by simple electrochemical diffusions, and were discussed in relation to an ionic permeation model assuming exchange permeations at certain sites.

Specific permeation is one of the most important properties of biological membranes. The permeation of small ions such as Na^+, K^+ or Cl^- is especially closely related to their physiological activities in the case of the neuronal membranes, and the mode of their permeation seems to be very important for the understanding of the properties of neuronal membranes.

In the case of the membranes of nerve-ending particles (synaptosomes), there have been a few investigations on passive ionic permeation (Marchbanks, 1967; Ling & Abdel-Latif, 1968; Keen & White, 1969, 1971). Marchbanks (1967) measured the permeability of Na^+, K^+ and Cl^- in hypertonic sucrose solution. Ling and Abdel-Latif (1968) studied mainly the active transport of Na^+, but reported a preliminary observation that ^{22}Na-efflux

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rate was dependent on salt concentrations of outside solutions. Keen and White (1969, 1971) studied passive flows of various substances into synaptosomes by the technique of the light-scattering measurement.

In the present study, passive ionic permeabilities were measured under various conditions by means of radioisotopic tracers and discussed in relation to an ionic permeation model.

Materials and Methods

Materials

^{22}Na was purchased as ^{22}NaCl (specific activity 14 C/mole) from the Radiochemical Centre, England; ^{42}K as ^{42}KCl (1.5 to 11 C/mole) from the Japan Atomic Energy Institute; ^{36}Cl as Na^{36}Cl (0.12 C/mole) from the Radiochemical Centre, England; D-[^{14}C]-galactose (35 C/mole) from Daiichi Pure Chemicals Co.; ouabain was obtained from Merck & Co., Inc.; membrane filters were purchased from Toyo Roshi Kaisha, Ltd. (type TM-2, pore size 0.45 μ, diameter 25 mm).

Preparation of Synaptosomes

Most of the procedure was performed following Keen and White (1971) whose method is based on that of Gray and Whittaker (1962). The whole brain of a female rat was homogenized in 15 ml 0.32 M sucrose with a Teflon glass homogenizer (clearance, about 0.2 mm; 800 rpm; 10 strokes). The homogenate was centrifuged at $1{,}000 \times g$ for 10 min, and the resulting supernatant was then centrifuged at $11{,}000 \times g$ for 20 min. The pellet was resuspended in 5 ml of 0.32 M sucrose solution and then subjected to a sucrose-density-gradient centrifugation, with a discontinuous density gradient consisting of 10 ml each of 0.8 M and 1.2 M sucrose. Following centrifugation in a Spinco SW-25 rotor with a Hitachi 40 P ultracentrifuge at $65{,}000 \times g$ for 90 min, the synaptosomal layer between 0.8 M and 1.2 M sucrose was separated with a bent-tip Pasteur pipette, diluted with an equal volume of 0.32 M sucrose, and centrifuged at $20{,}000 \times g$ for 30 min. The resulting pellet was resuspended in 1 ml of 0.32 M sucrose (or, when especially noted, sucrose solution with appropriate concentrations of NaCl or KCl whose tonicity was 0.32 osmolarity). The synaptosomal suspension thus obtained was used within 4 hr of preparation. The total time of preparation of the suspension was about 4.5 hr. The protein concentration of the suspension was 10.6 mg/ml in a typical case. The reproducibility of membrane preparation was examined regarding the ionic permeability. ^{22}Na-efflux curves into the standard outer solution (*see below*) were almost perfectly overlapped for every preparation.

Efflux Measurement

The procedure was similar to that of Kasai and Changeux (1971*a*). First, 0.1 ml of the synaptosomal suspension (0 °C) in 0.32 M sucrose (or saline sucrose solution when specifically noted) was added to 0.1 ml of a salt solution (0 °C) containing radioisotopes to form the "incubation solution." The composition of this incubation solution was

15 mM Tris-HCl buffer (pH 7.4), 1 mM Tris-phosphate buffer (pH 7.4), 3 mM glucose, 1.8 mM $MgCl_2$, 2.2 mM $CaCl_2$, and varying concentrations of NaCl, KCl and sucrose. The total tonicity of 2 [NaCl] + 2 [KCl] + [sucrose] was always at 0.28 osmolarity. The concentrations of radioisotopes $^{22}Na^+$, $^{42}K^+$, $^{36}Cl^-$, or D-[^{14}C]-galactose in the incubation solutions were 0.4 to 3 μC/ml. After the incubation at 0 °C for about 2 hr, the efflux measurement was started by diluting the incubation solution with 4.2 ml of a "dilution solution" (or an "outer solution"), which contained 15 mM Tris-HCl buffer (pH 7.4), 1 mM Tris-phosphate buffer (pH 7.4), 3 mM glucose, 1.8 mM $MgCl_2$, 2.2 mM $CaCl_2$, 0.1 mM ouabain, and various "test substances" (NaCl, KCl, LiCl, choline chloride, tetraethylammonium chloride, and/or sucrose). The total contribution of these test substances to the tonicity of each dilution solution was always 0.254 osmolarity. At given times, 1.0-ml samples were rapidly filtered on moist Toyo membrane filters and washed three times with 3 ml of cold (0 °C) washing solution which contained 5 mM NaCl, 5 mM KCl, 3.5 mM glucose, 2 mM $MgCl_2$, 18 mM Tris-HCl buffer (pH 7.4), 1 mM Tris-phosphate buffer (pH 7.4), and 0.27 M sucrose. The membrane filters were then dried and the radioactivities were counted. The counts obtained for the filters treated similarly without synaptosomes were used as background blank counts. The contribution due to ions bound to the synaptosome membrane was measured using osmotically ruptured synaptosomes, and was observed to be less than 10%. Thus, no special correction for these effects was made. The dilution solution was kept throughout at 18 °C unless specifically noted. The time elapsed between the initial pipetting and complete washing of the synaptosomes on the filter was within about 30 sec.

Uptake Measurement

The experiments were started by adding 0.2 ml of the synaptosomal suspension (0 °C) in 0.32 M sucrose to 0.3 ml of solution of radioisotopes (0 °C). The incubation was performed at 0 °C or 18 °C. At various times, 0.1-ml samples were removed from the reaction mixture, pipetted into 1.5 ml of ice-cold washing solution held on a membrane filter in a cylindrical filter holder. The mixture was immediately filtered, and washed three times as described above.

Counting of Radioactivity

The dried membrane filters were counted for 10 to 20 min with an Aloka TDS-1 crystal scintillation counter (γ-ray) with a channel width of #500, and a base line of #360, or with a Beckman LS-200B liquid scintillation counter (β-ray). The membrane filters were soaked in 10 ml of scintillation counting medium containing 4.0 g of PPO and 0.2 g of dimethyl POPOP in 1 liter of toluene. The differences between the counts obtained at the day of the experiment and those after about two weeks were attributed to ^{42}K, especially when doubly labeled with ^{22}Na and ^{42}K. By these methods, multiple labeling with ^{22}Na, ^{42}K and ^{36}Cl (or ^{14}C) could be discriminated.

Protein Determination

Protein was determined by the method of Lowry, Rosebrough, Farr and Randall (1951), using bovine serum albumin as the standard.

Results

Methods of Analysis of Efflux Data

Fig. 1 shows a few typical efflux curves of $^{22}Na^{+}$ ions from synaptosomes to diluting solutions of various conditions. The efflux rate varies with the condition of the solution. The efflux curves do not obey the simple first-order kinetics, as shown in the figure. These departures, whose reasons are ambiguous (*see* Discussion), give us difficulties in assessment of the permeability from the slopes of the curves. Avoiding these difficulties, we used two methods of analysis for quantitative treatments.

(*i*) *Apparent Half-Life Time* τ. This method was used in the investigations on the "microsacs" from electric eel, resulting in signal successes (Kasai & Changeux, 1971*a*). The precision of this method depends to a great extent on the extrapolated value of radioisotope content at zero time, and in our case cannot be attained. Therefore, we also used another method of analysis.

(*ii*) *The Second Method.* This method is based on the similarity of these efflux curves plotted semilogarithmically. First, ΔY^i was defined as the vertical distance between a point at time t on the i-th curve and a certain

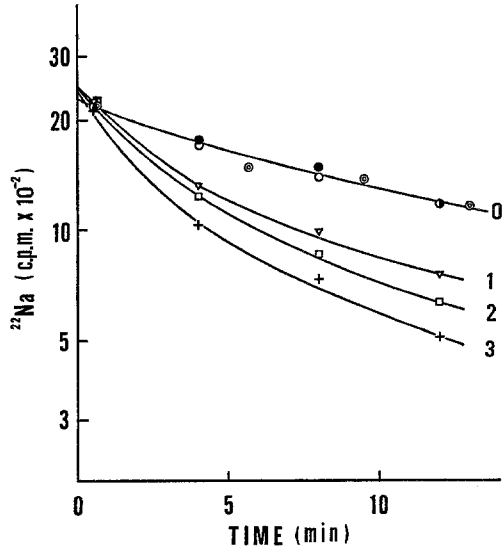

Fig. 1. ^{22}Na-efflux into various dilution solutions. The incubation solution was a standard one with 2 μC/ml ^{22}NaCl. Test substances of dilution were: •, 254 mM sucrose; ○, 127 mM choline chloride; ◎, 127 mM tetraethylammonium chloride; ▽, 20 mM NaCl + 107 mM choline chloride; □, 60 mM NaCl + 67 mM choline chloride; +, 127 mM NaCl

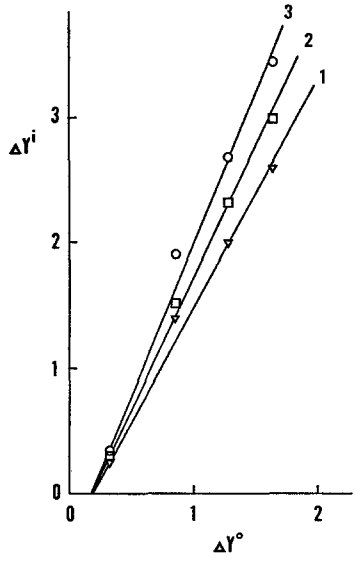

Fig. 2. Interrelations of efflux curves. ΔY^0 and ΔY^i were obtained from the data of Fig. 1. The lines 1, 2 and 3 correspond to the curves 1, 2 and 3 of Fig. 1, respectively. Both axes are expressed in an arbitrary unit

horizontal level, e.g., a tentative initial value. When ΔY^i of a curve was plotted against that of another, it was noticed that a fairly good straight line was obtained as shown in Fig. 2, at least within a certain initial period of time courses. The gradient of this straight line ω is independent of the horizontal level from which the vertical distance of each point was measured, and the following equations can be easily shown:

$$\omega=\frac{\partial(\Delta Y^1)}{\partial(\Delta Y^0)}=\frac{y^1}{y^0}=\left(\frac{dy^1}{dt}\right)_{t=0}\Big/\left(\frac{dy^0}{dt}\right)_{t=0}, \tag{1}$$

if the ratio of y for a curve to y for another is constant with regard to time; i.e.

$$y^1/y^0=\text{constant}, \tag{2}$$

where

$$y^i=\log[C^i(t)/C^i(0)], \tag{3}$$

$C^i(t)$ represents the content of radioisotopes in synaptosomes at time t, and the superscripts are for the identification of the curves.

This assumption was supported by the fact that fairly good straight lines which passed closely by the origin were obtained in Fig. 2. Thus, we

can obtain from such a gradient as ω a relative ratio of initial rates. As the second method of analysis, such gradients were used for the characterization of efflux curves. For this method, extrapolated initial values of radioisotope content are not important.

^{22}Na-Efflux

As shown in Fig. 1, the efflux rate of ^{22}Na ions varies with the conditions in the outer environment. It also varies with the condition of the intrasynaptosomal solution as is seen in Fig. 3. The shape of these efflux curves are similar to each other, and we can apply the second method of analysis mentioned above. The rate constants of ^{22}Na-efflux into various environments can be expressed in terms of ω; i.e., the efflux rate coefficient relative to that into a certain standard diluting solution, K. This factor K, however, varies with the intrasynaptosomal condition; i.e., that of the initial incubation. Experimentally, the relative ratio between K's, which we call α, is also determined with the same method of analysis as ω. Thus, we can analyze an efflux curve as follows. First, we define the standard intra- and extrasynaptosomal conditions. We take 254 mM sucrose solution with small amounts of Tris-HCl buffer and some other compounds as the standard outer solution and the ice-cold sucrose solution with 5 mM NaCl, 5 mM KCl, and small amounts of Tris-HCl buffer and some other compounds as the standard initial incubation solution (*see* Materials and

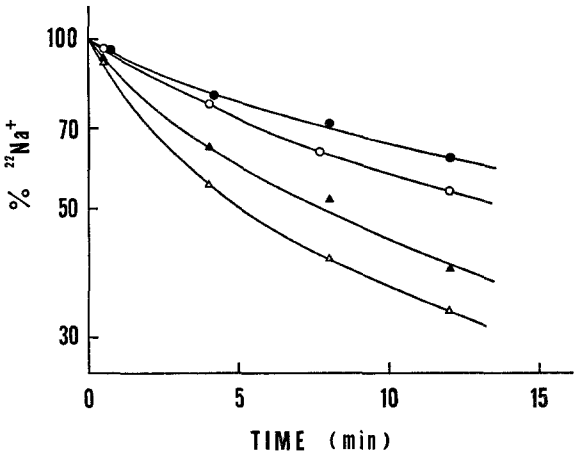

Fig. 3. Effects of concentrations of $[KCl]_{in}$ of incubation solutions on ^{22}Na-efflux rates. $[KCl]_{in}$ was 5 mM (○, △) or 100 mM (●, ▲). $[NaCl]_{in}$ was always 5 mM. Test substances of outer solutions were 127 mM choline chloride (○, ●) or 127 mM KCl (△, ▲)

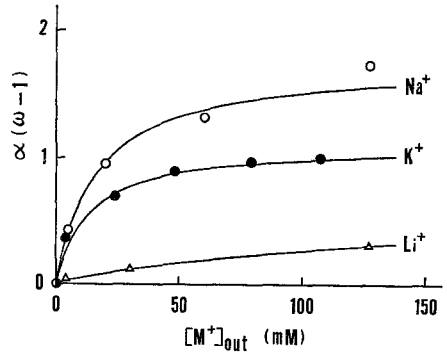

Fig. 4. Accelerations of ^{22}Na-efflux by monovalent cations in outer solutions. $\alpha(\omega-1)$ represents the apparent rate coefficient of the increased efflux [*see* Eq. (8)]. The initial incubation solution was the standard solution. Total tonicity and ionic strength of each outer solution were kept constant by adding appropriate concentrations of choline chloride. The solid curves for Na^+ and K^+ were calculated from Eq. (12) using values of Table 2

Methods). The efflux rate coefficient under these standard intra- and extrasynaptosomal conditions is defined as K^0. Second, we analyze each efflux curve obtained under various conditions. The analysis consists of two steps: (a) the comparison between the efflux curve to be analyzed and the standard curve under the same intrasynaptosomal condition which was measured in the standard outer solution; i.e., the determination of ω; and (b) the comparison between the standard curves, that is, between the one used in step (a) and the one under the standard intra- and extrasynaptosomal conditions; i.e., the determination of α. The efflux rate coefficient of the curve thus analyzed can be written as $\alpha K^0\omega$ or $\alpha\omega$ expressed in K^0 units.

(*i*) *Effect of Monovalent Cations in Outer Solutions.* The efflux rate was accelerated when sucrose of the outer solution was replaced by NaCl, while no change of the rate was observed when replaced by choline chloride or tetraethylammonium chloride (Fig. 1). This means that the efflux rate is independent of the ionic strength of the outer solution. The acceleration increased with the concentration of NaCl in the outer solution. This also holds for the cases of KCl and LiCl. The results of the quantitative analyses are illustrated in Fig. 4. The effect of ammonium ions was also examined. The efflux rates were measured in various concentrations of $[NH_4^+]_{out}$, keeping the sum of concentrations of $[K^+]_{out}$ and $[NH_4^+]_{out}$ constant (127 mM), but no change was observed in the rate. In other words, the effect of NH_4^+ in the outer solution on the acceleration of the efflux rate is

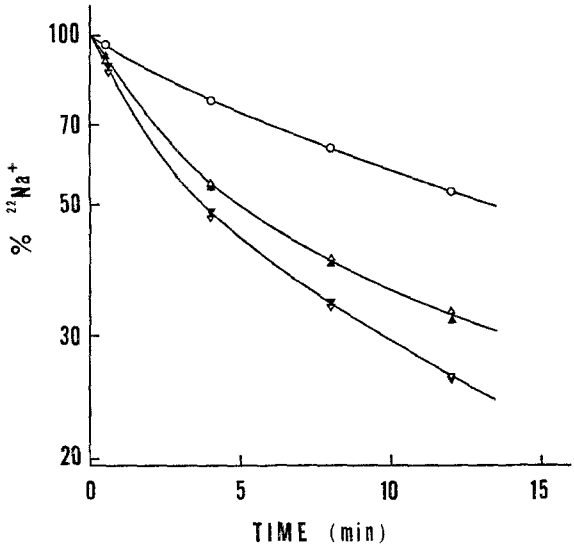

Fig. 5. Lack of effects of anions and osmotic pressure of outer solutions. Test substances of outer solutions were: ○, 127 mM choline chloride; ▲, 64 mM K_2SO_2 + 64 mM sucrose; △, 127 mM KCl; ▼, 60 mM NaCl + 67 mM choline chloride; ▽, 60 mM NaCl + 34 mM choline chloride. The initial incubation was performed under the standard condition

identical with that of K^+. From these results, the effects of various monovalent cations in the outer solutions on the acceleration of the efflux rate of ^{22}Na can be expressed as:

$$Na^+ > K^+ = NH_4^+ > Li^+ > \text{choline}^+ = \text{tetraethylammonium}^+ = 0. \quad (4)$$

(*ii*) *Effect of Anions in Outer Solutions.* As is already noted in the previous section, no change in efflux rates was observed by replacing sucrose in the outer solution with choline chloride or tetraethylammonium chloride. This result suggests that not only impermeably large cations but also anions such as Cl^- have no effect on the rate of Na^+-efflux. This holds for the case where the counter ions are permeable cations. The efflux rates into KCl solution or K_2SO_4 solution were measured, but no difference was observed (Fig. 5). It has been reported that synaptosomal membranes were impermeable to SO_4^{2-}, whereas they were fairly permeable to Cl^- (Keen & White, 1971). Our results show that neither permeable nor impermeable anions in outer solutions have any effects on Na-efflux.

(*iii*) *Effect of Monovalent Cations in Inner Solutions.* The efflux rate depends on the conditions of intrasynaptosomal solutions as well as those

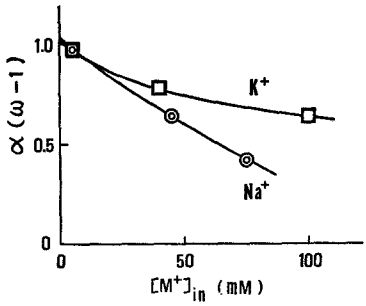

Fig. 6. Dependencies of ^{22}Na-efflux rate coefficient, $\alpha(\omega-1)$, on the concentrations of Na^+ or K^+ ions of incubation solutions. Outer solutions always contained 127 mM KCl as the test substance. ◎: Effect of $[Na^+]_{in}$. $[K^+]_{in}$ is always 5 mM. □: Effect of $[K^+]_{in}$. $[Na^+]_{in}$ is always 5 mM

of outer solutions, especially on the inner concentrations of monovalent cations. Fig. 6 shows the dependencies of the efflux rate coefficients on the concentrations of monovalent cations in the initial incubation solutions. It seems reasonable to consider that the concentrations $[Na^+]_{in}$ and $[K^+]_{in}$ of intrasynaptosomal solutions, at least the easily exchangable parts of them, are approximately equal to those of the initial incubation solutions (*see* the section titled "^{22}Na and ^{42}K Uptake"). On such an assumption, we could conclude from Fig. 6 that the efflux rate coefficient decreased as the concentration of $[Na^+]_{in}$ or $[K^+]_{in}$ increased.

(*iv*) *Effect of Osmotic Pressure of Outer Solution.* It has been known (Escueta & Appel, 1969; Keen & White, 1969; Kasai & Changeux, 1971*b*) that the vesicular membranes vary their volumes according to osmotic pressures of their environments which depend not only on the concentrations of solutes but also on the permeabilities of the solutes. Such increments of the volumes must be accompanied by some stretching stress on the membranes, which may affect their permeability properties. To examine this possibility, we measured the efflux rates in the outer solutions of different osmotic pressures, keeping the concentration of $[NaCl]_{out}$ constant. The result is shown in Fig. 5. When the osmotic pressure changed, the rate did not change if the concentration of $[NaCl]_{out}$ was kept constant. This finding supports the conclusion that the Na-efflux rate depends not on the osmotic pressure, the concentration of anions, or that of impermeable large cations such as choline, but on the concentration of permeable cations such as Na^+ or K^+ of the outer solution.

(*v*) *Effect of Temperature and Drugs.* The effect of temperature was studied by measuring efflux rates at 0 °C and 18 °C. The incubation solution

was the standard one, and the dilution solution contained 107 mM KCl and 20 mM tetraethylammonium chloride. Under these conditions, the ratio $\omega(18\,°C)/\omega(0\,°C)$ was observed to be 3.0. This value corresponds to $Q_{10}=1.9$ (activation energy of about 12 kcal/mole).

Effects of drugs on the ^{22}Na-efflux rate was examined under conditions similar to that for curve 1 of Fig. 1. Acetylcholine, epinephrine, norepinephrine, serotonine, γ-aminobutyric acid, glutamic acid, aspartic acid, glycine, or cyclic AMP, each at a concentration of about 1 mM, were examined. None of them was observed to affect the permeability of Na^+ ions.

^{42}K-Efflux

The efflux rate of ^{42}K was affected in similar ways to that of ^{22}Na. It was accelerated by permeable monovalent cations of the outer solution and was depressed by those of inner solution (Fig. 7). The shape of the efflux curves were not similar to each other, and the method of analysis used in the case of ^{22}Na could not be applied. The first method mentioned above, the apparent half-life time τ was used. The results by this method of analysis are given in Table 1 together with the results for ^{22}Na-efflux treated in the same manner. We could summarize the results concerning the ^{42}K-efflux as follows:

(*i*) The acceleration of ^{42}K-efflux by monovalent cations in outer solutions was much larger in the case of K^+ ions than the others. The order

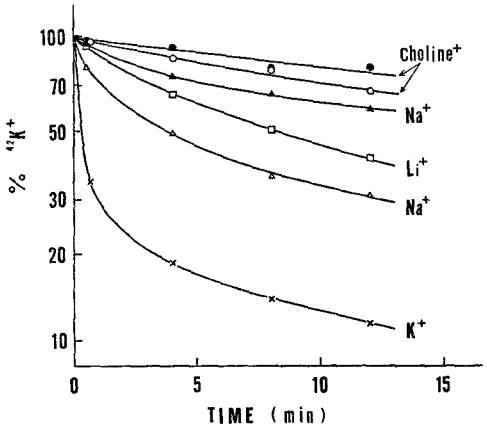

Fig. 7. Effects of monovalent cations of outer solutions on ^{42}K-efflux. The outer solutions contained as test substances 127 mM chlorides of various monovalent cations indicated in the figure. Initial incubation solutions of the case of • and ▲ contained 5 mM NaCl and 65 mM KCl. For other cases the standard condition was used

Table 1. Effects of outer solutions on ^{42}K-, ^{22}Na-, and ^{36}Cl-efflux rates

Efflux	Outer solution[a]	Half-life time (τ)	Degree of acceleration $\left(\frac{\tau_0}{\tau}-1\right)$
^{42}K-Efflux	254 mM sucrose	23 min ($=\tau_2$)	0
	127 mM Choline chloride	23	0
	127 mM LiCl	8	1.9
	127 mM NaCl	4	4.8
	5 mM KCl + 122 mM Chol Cl	4	4.8
	5 mM KCl + 122 mM NaCl	2.5	8.2
	10 mM KCl + 117 mM NaCl	1.5	14
	20 mM KCl + 107 mM Chol Cl	1	22
	30 mM KCl + 97 mM Chol Cl	0.5	45
	127 mM KCl	0.5	45
^{22}Na-Efflux	254 mM sucrose	13 ($=\tau_0$)	0
	127 mM Chol Cl	13	0
	127 mM NaCl	3	3.3
^{36}Cl-Efflux	254 mM sucrose	13 ($=\tau_0$)	0
	127 mM KCl	13	0

[a] Outer solutions always contain 15 mM Tris-HCl buffer (pH 7.4), 1 mM Tris-phosphate buffer (pH 7.4), 3 mM glucose, 1.8 mM $MgCl_2$, 2.2 mM $CaCl_2$, and 0.1 mM ouabain in addition to the "test substances" mentioned in this column.
Initial incubation solutions were the standard one for all cases.

of this effect was as follows:

$$K^+ \gg Na^+ > Li^+ > \text{choline}^+ = 0. \tag{5}$$

This effect of $[K]_{out}$ on the efflux rates was impossible to measure at a concentration higher than $[K]_{out}$ of 30 mM by the filtration technique.

(*ii*) The maximum rate and the maximum degree of acceleration $\left(\frac{\tau_0}{\tau}-1\right)$ of ^{42}K-efflux were much larger than those of ^{22}Na-efflux. This finding accorded well with the fact that biological membranes are generally much more permeable to K^+ ions than to Na^+ ions.

(*iii*) When there were no permeable monovalent cations in outer solutions, e.g., in the case of the standard outer solution, ^{42}K ions seemed to flow out more slowly than ^{22}Na ions. (In this case, the efflux of ^{42}K or ^{22}Na must have been accompanied with effluxes of anions such as Cl^-.)

As for the last point mentioned above, there are some ambiguities. Although the effect of the active transport can be neglected (the effluxes were measured with ouabain in outer solutions), that of the inner concentrations of Na^+ and K^+ must not be neglected. As noted above, both ^{22}Na-efflux and ^{42}K-efflux are dependent on them, and are depressed with increasing $[Na^+]_{in}$ and $[K^+]_{in}$. Because the inner concentrations, $[Na^+]_{in}$ and $[K^+]_{in}$, might fluctuate to some extent, a definite conclusion about this point cannot be drawn.

^{36}Cl- and ^{14}C-Galactose-Efflux

As described above, the efflux rates of the monovalent cations such as Na^+ or K^+ changed as the ionic conditions of the outer solutions varied. To investigate whether these changes were accompanied by the changes of the efflux rates of other materials such as monovalent anions or uncharged molecules, we measured the rates of ^{36}Cl and D-[^{14}C]-galactose under similar conditions to those under which the rates of monovalent cations were measured. The results, shown in Fig. 8, indicated that the efflux rates of ^{36}Cl and D-[^{14}C]-galactose were independent of the ionic environments. Galactose may not necessarily be metabolically inactive for synaptosomes

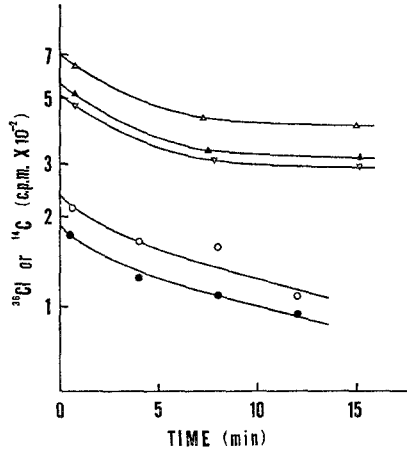

Fig. 8. Absence of effects of outer solutions on efflux rates of D-[^{14}C]-galactose (△, ▲, or ▽) and ^{36}Cl (○ or ●). Initial incubation solutions for the cases of ^{14}C-galactose contained 100 mM NaCl, 9 mM KCl and 2.3×10^{-8} M galactose (0.8 μC/ml). Those for the cases of ^{36}Cl were the standard solution with 0.6 μC/ml ^{36}Cl. Test substances of the outer solutions were: ▲, 10 mM KCl + 117 mM tetraethylammonium chloride (TEA Cl); △, 40 mM KCl + 87 mM TEA Cl; ▽, 100 mM KCl + 27 mM TEA Cl; ○, 254 mM sucrose; ●, 127 mM KCl. In the cases of D-[^{14}C]-galactose, washing solution contained 10 mM D-galactose in addition to the composition described in Materials and Methods

(Quastel, 1970), and the nature of its efflux may not be similar to that of inorganic ions, but at least, it may be concluded that the efflux of galactose does not change when those of monovalent ions change, and that the former is independent of the latter. The same is also the case for ^{36}Cl-efflux. The efflux rate of Cl^- ions was also observed to be very close to that of Na^+ ions under the standard condition (Table 1). In view of these findings, we can exclude such a possibility that the membrane structure was made loose by the change of ionic environment, resulting in permeability change for all kinds of permeants.

^{22}Na and ^{42}K Uptake

For our investigations, it was necessary to control the intrasynaptosomal concentrations of Na^+ and K^+. It is difficult to determine them exactly, because the volume of the intrasynaptosomal space is difficult to know in spite of some efforts (Marchbanks, 1967; Escueta & Appel, 1969), because it is uncertain whether the exchanges between inner and outer ions is complete or not (Weinstein, Varon, Muhleman & Roberts, 1965), and because the presence of the Na-pump (Albers, Rodriguez de Lores Arnaiz & De Robertis, 1965; Kurokawa, Sakamoto & Kato, 1965) may hinder the complete equilibration of monovalent cations between inner and outer solutions. Thus, we compromised to take them as equal to the concentrations of the initial incubation solutions. To examine this assumption, we measured the uptake of Na^+ and K^+ ions under similar conditions to those which were adopted in the initial incubations. The results are shown in Fig. 9. Under the typical three conditions of Na^+ and K^+ concentrations, the uptake of the radioactive ^{22}Na ions was of the same order of magnitude if the concentrations of ^{22}Na in the incubation solutions were the same in spite of the fact that the specific activities of Na^+ were varied by about 20-fold. This also held for the case of K^+. At this temperature, the Na, K-ATPase activity must be mostly suppressed. Moreover, the amount of radioisotopes reached a nearly constant level after a certain period of incubation, meaning that the distributions of the hot and cold ions between inner and outer solutions were at a stationary state. These facts indicate that the amounts of Na^+ or K^+ ions retained in the synaptosomes are at least roughly proportional to the outer concentrations of $[Na^+]_{out}$ or $[K^+]_{out}$. In addition, after incubation for 70 min at 0 °C under the same condition as that for curve 1 of Fig. 9(*a*), the ratio of the apparent K^+ equilibrium volume of the synaptosomes (volume obtained under the assumption that ^{42}K ions are equilibrated between inner and outer solutions) *vs.* that of Na^+ was 1.5:1. Taking all these findings into account, we could fairly well

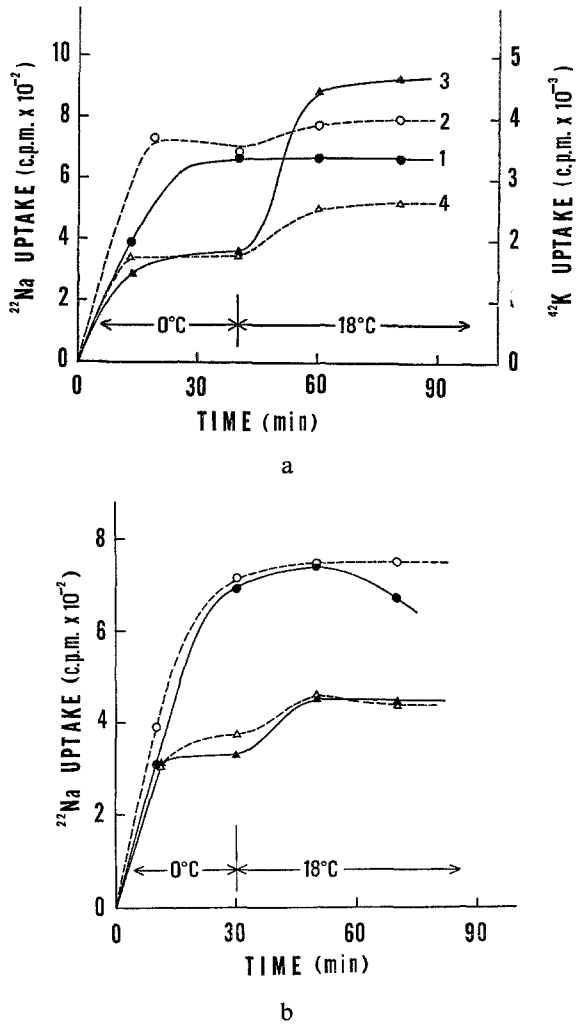

Fig. 9. (*a*) Uptakes of ^{22}Na (curves 1 and 2) and ^{42}K (curves 3 and 4) into the synaptosomes. The concentrations of $[NaCl]_{in}$ and $[KCl]_{in}$ of the solution in which synaptosomes were incubated were 71 mM and 4.4 mM, respectively, with (------) or without (———) 0.5 mM ouabain. The concentrations of radioisotopes were: ^{22}Na, 0.7 μC/ml; ^{42}K, about 0.6 μC/ml (the value at the time of counting), for all curves. (*b*) Uptake of ^{22}Na into synaptosomes. The conditions of incubation were: ○ or ●, $[NaCl]_{in} = 5$ mM, $[KCl]_{in} = 5$ mM; △ or ▲, $[NaCl]_{in} = 5$ mM, $[KCl]_{in} = 88$ mM; with (------) or without (———) 0.5 mM ouabain. ^{22}Na concentration was 0.6 μC/ml for each curve

assume that the intrasynaptosomal concentrations of Na^+ and K^+ were at least roughly equal to those of the incubation media after sufficient periods of incubation at 0 °C.

Ionic Permeation Model

As is discussed later, the changes in the cationic permeation observed here are difficult to explain from the standpoint of the simple electrochemical diffusion (*see* Discussion). They can be explained by a model which is based on the exchange between the monovalent cations of inner and outer solutions similar to the case of Ca^{2+} of erythrocyte membranes (Porzig, 1970).

The efflux of Na^+ ions, j_{Na}, can be written as:

$$j_{Na}=\alpha K^0 \omega [Na^+]_{in}, \tag{6}$$

where K^0 represents the efflux rate coefficient of Na^+ ions under the standard intra- and extrasynaptosomal conditions, α and ω represent the effects of intra- and extrasynaptosomal conditions, respectively, which are both unity under the standard conditions. The efflux j_{Na} can be separated into two parts:

$$j_{Na}=\alpha K^0 [Na^+]_{in}+\alpha K^0(\omega-1)[Na^+]_{in}. \tag{7}$$

The first term represents the efflux into the standard outer solution, which is accompanied by an equivalent number of anions, mainly Cl^- ions, and the second term represents the increased efflux when the outer sucroses are replaced by monovalent cations. The latter can also be written in K^0 units (we measured all the rates in K^0 units in this paper) as:

$$j_{Na}^{(inc)}=\alpha(\omega-1)[Na^+]_{in}. \tag{8}$$

This component of the efflux is accompanied by the influx of cations, and it is reasonable to assume certain sites at which the exchange between monovalent cations takes place (Fig. 10). Each association "hand" of the site, whose total amount is n, is in association equilibrium with the i-th

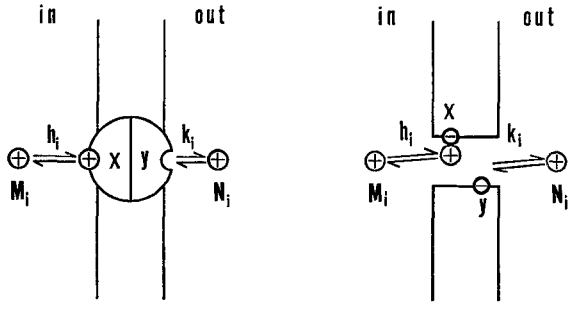

Fig. 10. Schematic representation of a specific site of exchange permeation

monovalent cation of the inner or outer solution, whose total amount is M_i or N_i, respectively:

$$\begin{aligned} \text{inside: } h_i &= \frac{n x_i}{n\left(1-\sum_i x_i\right)(M_i - n x_i)} \simeq \frac{x_i}{\left(1-\sum_i x_i\right) M_i}, \\ \text{outside: } k_i &= \frac{n y_i}{n\left(1-\sum_i y_i\right)(N_i - n y_i)} \simeq \frac{y_i}{\left(1-\sum_i y_i\right) N_i}, \end{aligned} \tag{9}$$

where h_i (or k_i) is the association constant between the hands inside (or outside) and the i-th monovalent cations. x_i (or y_i) is the fraction of hands inside (or outside) which are associated with the i-th monovalent cations, and the summations are for all kinds of monovalent cations present. The last expressions of these equations are derived from the assumption that $M_i \gg n x_i$ (or $N_i \gg n y_i$). From Eq. (9), x_i and y_i can be solved:

$$\begin{aligned} x_i &= h_i M_i / \left(1+\sum_j h_j M_j\right), \\ y_i &= k_i N_i / \left(1+\sum_j k_j N_j\right). \end{aligned} \tag{10}$$

The increased efflux of Eq. (8) can be given by the expression:

$$j_i^{(\mathrm{inc})} = n x_i \left(\sum_j s_{ij} y_j\right), \tag{11}$$

where s_{ij} represents the rate constant of the exchange between the i-th and the j-th monovalent cations bound to hands inside and outside, respectively. From Eqs. (8), (10) and (11) we obtain

$$\alpha_i(\omega_i - 1) = \frac{n h_i \left(\sum_j s_{ij} k_j N_j\right)}{\left(1+\sum_j h_j M_j\right)\left(1+\sum_j k_j N_j\right)}. \tag{12}$$

If there is only one kind of permeable monovalent cation in the outer solution, Eq. (12) can be rewritten

$$\frac{1}{\alpha_{\mathrm{Na}}(\omega_{\mathrm{Na}} - 1)} = \frac{1+\sum_j h_j M_j}{n s_{\mathrm{Na},j} h_{\mathrm{Na}}} \left(1 + \frac{1}{k_j N_j}\right). \tag{13}$$

In Fig. 11, the experimental data are plotted in this manner with N_j re-written in concentration units, which actually give fairly good straight lines.

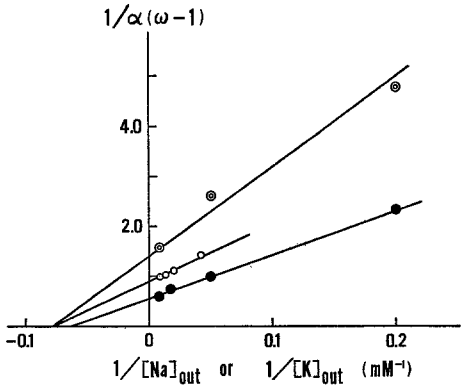

Fig. 11. Lineweaver-Burke plot of ^{22}Na-efflux kinetics. ◎: Effect of $[K^+]_{out}$ when initially incubated in the solution which contained 100 mM KCl and 5 mM NaCl. ○: Effect of $[K^+]_{out}$ when initially incubated in the standard incubation solution. ●: Effect of $[Na^+]_{out}$ when incubated in the standard solution

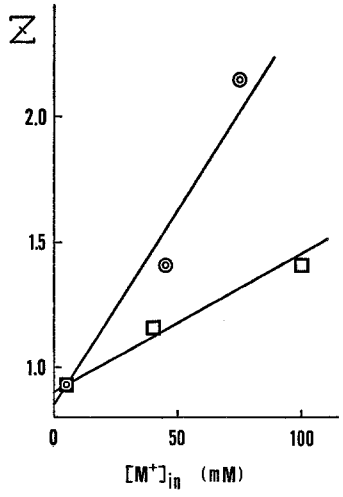

Fig. 12. Effects of monovalent cations of incubation solutions on ^{22}Na-efflux rates (replotted data of Fig. 6). Z is defined as the left-hand side of Eq. (14). ◎: Effect of $[Na^+]_{in}$. $[K^+]_{in}$ is always 5 mM. □: Effect of $[K^+]_{in}$. $[Na^+]_{in}$ is always 5 mM

From this figure, k_j and $s_{Na,j}/s_{Na,j'}$ could be obtained. On the other hand, when K^+ ions are the only permeable monovalent cations present in the outer solution and if we neglect monovalent cations inside the membranes except Na^+ and K^+, Eq. (12) can be transformed into Eq. (14) (amounts of

Table 2. A set of parameters of the model[a]

	Na^+ ($i=1$)	K^+ ($i=2$)
Outside association constant, k_i	$k_1=6.4\times10^{-2}$ mM^{-1}	$k_2=7.4\times10^{-2}$ mM^{-1}
Inside association constant, h_i	$h_1=2\times10^{-2}$ mM^{-1}	$h_2=0.7\times10^{-2}$ mM^{-1}
Exchange rate coefficient, s_{ij}[b]	$ns_{11}=1.0\times10^2$	$ns_{12}=6.4\times10$ ($s_{11}/s_{12}=1.6$)

[a] Because of the difficulty of determining intrasynaptosomal concentrations of $[Na^+]_{in}$ and $[K^+]_{in}$, values determined from Fig. 12 (h_1, h_2, and ns_{11} or ns_{12}) are less reliable than those determined from Fig. 11 (k_1, k_2, and the ratio s_{11}/s_{12}).
[b] Expressed in K^0 units.

Table 3. Comparison of observed and calculated values of $\alpha(\omega-1)$

Concentrations in outer solution		Observed values of $\alpha(\omega-1)$	Calculated values of $\alpha(\omega-1)$
$[Na^+]_{out}$ (mM)	$[K^+]_{out}$ (mM)		
5	5	0.74	0.58
30	30	1.02	1.14
64	64	1.37	1.27

ions are expressed in concentration):

$$\frac{(s_{Na,Na}/s_{Na,K})\,k_{Na}[K^+]_{out}}{\alpha_{Na}(\omega_{Na}-1)\,(1+k_{Na}[K^+]_{out})}\equiv Z$$
$$=\frac{1}{n\,s_{Na,K}}\left\{\frac{1}{h_{Na}}+[Na^+]_{in}+\left(\frac{h_K}{h_{Na}}\right)[K^+]_{in}\right\}. \tag{14}$$

The left-hand side of this equation can be determined experimentally, and is called Z. Fig. 12 gives the experimental data plotted in the manner of Z *vs.* M_j. From Figs. 11 and 12 a set of parameters was obtained, which is shown in Table 2. The solid curves for $[Na^+]_{out}$ and $[K^+]_{out}$ of Fig. 4 were calculated from Eq. (12).

The model was also applied to the case where both Na^+ and K^+ ions are present in outer solutions. The calculated values of $\alpha(\omega-1)$ are given in Table 3 with corresponding values obtained experimentally. These results seem to support the model described in this section.

Discussion

The radioisotopic effluxes measured in this study appeared to deviate from the first-order kinetics. Such deviations were also observed in the case of "microsacs" from the electric eel (Kasai & Changeux, 1971*a*, *b*). These deviations were observed in a stationary state as well; i.e., under the condition that the intra- and extrasynaptosomal solutions were the same. They might be interpreted on the basis of heterogeneity of synaptosomes, both in size (Clementi, Whittaker & Sheridan, 1966) and nature (Iversen & Snyder, 1968; Kuhar, Shaskan & Snyder, 1971), in a way similar to the case of microsacs. In the case of synaptosomes, moreover, it must be considered that the mitochondria contained in the synaptosomal preparations make them double-compartment systems arranged both in parallel and in series, and that all of the intrasynaptosomal K^+ ions were not rapidly exchangable (Weinstein & Kuriyama, 1970). Because of these various reasons, the efflux curves did not follows a simple exponential law, and two methods of analysis were used (*cf.* Materials and Methods). Without exact knowledge about the reasons which caused the deviations, the two methods are difficult to correlate quantitatively. The results of ^{42}K-effluxes could not be treated so extensively as those of ^{22}Na-effluxes in the present paper.

The efflux rates of ^{22}Na and ^{42}K were found to depend on the ionic compositions both inside and outside the synaptosomes. As the interpretation of this dependency, there may be three possible mechanisms: (*i*) simple diffusion of each ionic species whose efflux rate depends on the electric potential across the membrane; (*ii*) exchange permeation between ions inside and outside the membrane through a certain specific site (or "channel"); (*iii*) permeation through a "channel" whose permeability changes according to the environmental condition. The first and the second mechanisms interpret the changes of the efflux rates by means of variations of properties of environments and not of the membrane, while the third attributes them to the changes of properties of the membrane itself.

Of these three mechanisms, the first one could be ruled out as discussed in the following. First, it is difficult to explain from this standpoint the observation that easily permeable anions such as Cl^- did not affect the efflux rates of monovalent cations at all. Second, in contrast to the expectation from this mechanism, ^{36}Cl-efflux rate was observed not to be affected by the changes of ionic conditions which affected both ^{22}Na- and ^{42}K-efflux rates. And finally, the order of the acceleration effect of various monovalent cations on the efflux rate of ^{22}Na was not the same as that of ^{42}K [Eqs. (4) and (5)], although they must coincide with each other according to the mechanism (*i*).

The permeation model described above belongs to the second mechanism, and it is to be emphasized that there is some kind of interaction (or selectivity) between the entering and outgoing cations. In other words, the model in which the exchange takes place in such a manner that entering and outgoing ions traverse independently of each other keeping the electric neutrality, belongs to the first mechanism, and could be ruled out. It is not verified if the transmembrane potential varied with concentrations of $[K^+]_{out}$ or $[Cl^-]_{out}$. It could be taken to be zero or negligible at equilibrium (Marchbanks, 1967), but it seems to be rather natural in the case of the present experiments to assume that some potential difference existed immediately after the dilution and during the early period of the efflux process. At any rate, because of the reasons discussed above, it would be reasonable to assume that the transmembrane potential had little or no relation to the efflux rates of ions studied here as well as that of uncharged substances. Thus, the model described in the previous section does not take account of such an effect.

It is true that the third mechanism which assumes some changes of the membranes cannot be excluded. Kasai and Changeux (1971*b*) reported that the membrane permeability for only small cations changed and the permeability for negatively charged or uncharged permeants did not. This kind of possibility cannot be ruled out from the present case. However, if we use assumption (*iii*), the discrepancy of Eqs. (4) and (5) requires rather implausible change in the permeability of the membranes. Thus, the third mechanism was not used.

From the results and using the permeation model, some knowledge could be obtained about the properties of the synaptosomal membranes in regard to the permeation of various ions. The permeation of monovalent cations such as Na^+ or K^+ has two modes: leakage as a neutral salt such as NaCl or KCl, and exchange between monovalent cations of inner and outer solutions.

The fact that s_{11} differed from s_{12} (Table 2) could be interpreted that the ionic selection was performed not only for entering and outgoing ions independently but also for a pair of entering and outgoing ions.

^{36}Cl-efflux rate was observed to be independent of the ionic condition of the outer solution (Fig. 8). It was also found that the rate was very close to that of ^{22}Na-efflux under the standard condition (Table 1). These observations suggest that the observed ^{36}Cl-efflux is that accompanied by monovalent cations; i.e., leakage as a neutral salt such as NaCl, and that there are no special "channels" for anions in the synaptosomal membranes.

This might mean that anions such as Cl^- played no direct part in electrophysiological activities of synapses of rat brains (Kuriyama & Roberts, 1971).

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