Movements of Radioactive Sodium in Cerebral-Cortex Slices in Response to Electrical Stimulation

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1. Sodium exchange was measured with ^{24}Na in incubated guinea-pig cerebral-cortex slices maintained under adequate metabolic conditions with a steady content of fluid and ions resembling that of brain *in vivo*. 2. Evidence was obtained indicating that Na^+ ions behaved in the inulin space as if they were extracellular, and that their entry into the non-inulin space of unstimulated tissue was about 10 times slower and could be separated, on the basis of complete exchangeability, into two components, a 'fast' one, which reacted to electrical stimulation, and a 'slow' one, exchanging at a rate of about 8 μequiv./g./hr., which was not affected by stimulation. 3. The average rate of sodium turnover in unstimulated slices was 175–275 μequiv./g./hr., whereas that for stimulated slices was approx. 4–6 times this, or 1050–1180 μequiv./g./hr. The stimulated rate was equivalent to a turnover of 32% of the sodium in the non-inulin space/min., or 3 mμequiv./g./impulse. 4. Response to the onset of stimulation appeared to be immediate, but after cessation of stimulation increased sodium movements persisted for several minutes before return to unstimulated values. 5. Calculations based on electrochemical gradients suggested that about one-quarter of the energy available from respiration was required for sodium and potassium transport at maximal rates in both unstimulated and stimulated cerebral-cortex slices.

The changes in ionic gradients that occur during electrical activity in excitable tissues (Hodgkin, 1951, 1958; Eccles, 1957; Shanes, 1958) have provided the basis for the interpretation of ion movements in electrically stimulated cerebral tissues *in vitro* (McIlwain, 1963). Direct evidence in cerebral-cortex slices for this interpretation includes the gain of sodium and loss of potassium that occur in response to electrical stimulation (Bachelard, Campbell & McIlwain, 1962), the restoration of pre-stimulation values for the ionic gradients after termination of pulses (Keesey, Wallgren & McIlwain, 1965), and the changes in resting potentials of individual cells in cerebral-cortex slices that parallel these changes in ion content (Hillman, Campbell & McIlwain, 1963). The turnover of potassium in cerebral-cortex slices has also been measured *in vitro* with ^{42}K (Krebs, Eggleston & Terner, 1951) and the movement of ^{42}K both into and out of isolated cerebral-cortex slices has been found to increase during only a few seconds of electrical stimulation (Cummins & McIlwain, 1961).

Though measurements have been made of diffusion of radioactive sodium from the extracellular spaces of thick blocks of mammalian cerebral cortex of doubtful metabolic status (Garoutte & Aird, 1956; McLennan, 1957), studies *in vitro* of sodium exchange in the cellular elements of cerebral cortex have encountered technical and analytical difficulties. Not the least of these has been the problem of maintaining cerebral tissue during incubation with a constant fluid and ion content closely resembling that which occurs *in vivo* (Keesey *et al.* 1965). Further, the relatively high physiological concentration of external sodium required for this maintenance (Pappius, Rosenfeld, Johnson & Elliott, 1958; Bachelard *et al.* 1962) tends to obscure small changes in sodium concentration that may occur intracellularly. For this reason and because of the cellular heterogeneity and complexity of cerebral cortex, it has become especially important to have a reliable measure of the portion of this tissue in which ions behave as if they were extracellular.

The work reported below represents a description of the movements of radioactive sodium in incubated mammalian cerebral-cortex slices maintained *in vitro* under adequate metabolic conditions with a steady content of fluid and ions. It includes

evidence indicating that Na^+ ions behave in the inulin space as if they were extracellular and that sodium turnover in the non-inulin space can be separated into two components, a 'fast' one, increasing on electrical stimulation, and a 'slow' one, which is not affected by stimulation.

EXPERIMENTAL

As described in detail by Keesey *et al.* (1965), slices of guinea-pig cerebral cortex 0·35 mm. thick, weighing 35–65 mg., were obtained with a dry blade and guide within 1·5–5·0 min. of stunning the animal and were placed immediately into glucose–bicarbonate medium at 37°. Each slice was then taken up from this medium in a rapid-transfer holder and before any further procedures were carried out was incubated for at least 30 min. in a 30 ml. beaker containing 5 ml. of glucose–bicarbonate medium containing inulin (1%) through which was bubbled a gas mixture of $O_2 + CO_2$ (95:5).

^{24}Na *influx*. Movement of ^{24}Na into slices during gradated intervals of 1 to 60 min. was obtained as follows. By means of a 1 ml. syringe and a long needle, 0·1–1·0 ml. of glucose–bicarbonate medium in which a calculated portion of the $^{23}NaCl$ content had been replaced by $^{24}NaCl$ (obtained as an iso-osmotic solution from The Radiochemical Centre, Amersham, Bucks.) was injected at 'zero time' into the incubation medium to give a radioactivity concentration of 1·5 μc/ml. without changing either the osmotic or ionic composition of the medium. Mixing was accomplished by gentle rotation of the rapid-transfer holder in the beaker and by the bubbling of the gas mixture through the medium. At a defined time from 1 to 60 min. after injection each slice was rapidly released from its holder into its medium, picked out with a bent-wire rider within 3–6 sec. of release, and reweighed.

Each slice after being reweighed was ground in 4 ml. of 6% (w/v) trichloroacetic acid and the suspensions were allowed to stand for 30 min. at room temperature before being centrifuged at 800*g* for 10 min. Each of the supernatant fluids was poured into a 10 ml. volumetric flask. The residues were each suspended in 5 ml. of glass-distilled water, centrifuged again and the supernatants added to their respective flasks, which were then diluted to 10 ml. with glass-distilled water and shaken. No measurable radioactivity was found in further resuspensions of the residues in water. A 50 μl. sample of each radioactive medium from which a slice had been removed was added to 4 ml. of glass-distilled water and, by treatment identical with that described for the tissue samples, was diluted to 10 ml. in a volumetric flask for the determination of radioactivity described below.

^{24}Na *efflux*. Movement of ^{24}Na out of slices during gradated intervals of 1 to 60 min. was measured on slices that had been incubated during the initial period of 30–60 min. after slicing in 5 ml. of glucose–bicarbonate medium in which a calculated portion of the $^{23}NaCl$ had been replaced by $^{24}NaCl$ to give a radioactivity concentration of 4 μc/ml. (estimated tissue dose about 200 rads). Several methods of following the subsequent outflow of ^{24}Na from these slices into non-radioactive medium were attempted, but none proved completely reliable and reproducible.

To obtain conditions of ^{24}Na efflux comparable with those under which the measurements of ^{24}Na influx described above were performed, the following method was adopted. After incubation for 30 min. in ^{24}Na-containing glucose–bicarbonate medium, each slice with its holder was rinsed twice in 7 ml. of non-radioactive medium for 30 sec. to remove radioactivity from the holder before being transferred finally at 'zero time' to a beaker containing exactly 5·0 ml. of non-radioactive medium, from which 0·1–0·2 ml. samples were removed by weighed plastic syringes at defined times from 0·5 to 10 min. after 'zero time'. Similar samples were also taken by syringe from each rinse and from the radioactive solution in which the slice had originally been incubated.

After all samples had been obtained from its medium, each slice was released from its holder into the medium, picked out with a bent-wire rider, reweighed, homogenized, and the water-soluble contents of the slice diluted to 10 ml. as described above for the influx experiments. Each syringe containing a sample of medium was reweighed to obtain a measure to the nearest microlitre of the volume removed, and then each of these samples was diluted to 10 ml. with distilled water (except the original incubation medium, which was diluted to 100 ml.) for determination of radioactivity as described below.

Electrical stimulation. Movements of ^{24}Na into and out of cerebral-cortex slices were investigated under four experimental situations: (*a*) ^{24}Na movements in slices that received no electrical stimulation; (*b*) ^{24}Na movements during the initial response to electrical stimulation, in which stimulation was begun at 'zero time' (i.e., for influx, at the time of injection of ^{24}Na-containing solution into the medium surrounding the slice, and, for efflux, at the time the slice and holder were transferred from the second rinse to the final beaker from which samples were to be removed) and continued until termination of the experiment; (*c*) ^{24}Na movements during a steady-state response to electrical stimulation (Keesey *et al.* 1965), in which electrical stimulation was applied for 10 min. before 'zero time' and continued throughout the experiment; (*d*) ^{24}Na movements during recovery after cessation of electrical stimulation, in which electrical stimulation was applied for 10 min. before being turned off at 'zero time'.

Electrical stimulation was applied through silver grids in the rapid-transfer holders in the form of condenser pulses of alternating polarity and exponential time–voltage relationship with a peak potential of 10 v, a time constant of 0·4 msec. and a frequency of 100 pulses/sec. (McIlwain, 1954).

Measurement of radioactivity. The radioactivities of the slices and of all samples of media were determined in succession by using the same 10 ml. well-type Geiger–Müller tube for liquid samples (type M-6H; 20th Century Electronics Ltd., New Addington, Surrey) and an Ekco model N530F scaler. All counts were corrected for background activity and for radioactive decay from the beginning of the counting period. Each slice from an influx experiment and the sample of its incubation medium were counted twice for 5 min. (at least ten times background) and the results averaged. The coefficient of variation for radioactive measurement of eight samples of identical radioactive medium, used for incubation of eight different slices, was 2% for one influx experiment and 4% for another. The samples from an efflux experiment were counted only once and were usually about five times background.

Analytical methods. After its amount of radioactivity had been determined, the 10 ml. sample for each tissue slice was poured from the Geiger–Müller counting tube into a 25 ml. volumetric flask. The Geiger–Müller tube was repeatedly rinsed out with small amounts of glass-distilled water, which were added to the contents in the flask, and each flask was diluted to 25 ml. with glass-distilled water. After a suitable time had been allowed for radioactive decay, all tissue samples and samples of pooled media, standards and blank were analysed for sodium, potassium, chloride and inulin as described by Keesey *et al.* (1965). Samples of pooled media from each experiment and not the samples of media used for radioactivity determinations were the ones analysed for the ion and inulin content in the medium.

Expression of results. All quantities of ions are expressed as μequiv./g. initial wt. of tissue, and the inulin space and non-inulin space are expressed as μl./g. initial wt., as explained by Keesey *et al.* (1965).

Influx. Assuming that the concentration gradient of radioactivity between external medium and tissue was the driving force for movement of ^{24}Na into the slice and that ^{23}Na and ^{24}Na behaved identically, the amount of sodium exchanged with the slice is given by C/A_1 μequiv./g., where C is the counts/min./g. of slice and A_1 is the specific activity of the sodium in the incubation medium (counts/min./μequiv. of sodium). If the sodium of the inulin space was of the same specific activity as the sodium of the incubation medium and I is the sodium content (μequiv./g.) in the inulin space, then the sodium exchanging with the non-inulin space may be given by the expression C/A_1-I, and that not exchanged by $T-(C/A_1-I)$, where T is the total sodium content (μequiv./g.) of the non-inulin space.

Efflux. Because of the considerable but variable amount of radioactivity on the holders that even after two rinses entered the sampled medium during the first 0·5 min., the results of efflux experiments have not been expressed in terms of μequiv. of sodium/g. of tissue but by the fraction $(R-X_t)/R$. R is the total amount of radioactivity (counts/min.) transferred to the beaker at 'zero time' and is calculated from the sum of radioactivities in beaker and in slice at the end of the experiment plus the amount of radioactivity removed by all sampling. X_t is the amount of radioactivity (counts/min.) having entered the medium at time t and is calculated from the concentration of radioactivity in the sample at time t multiplied by the total volume in the beaker at time t, plus all previously removed radioactivity. Therefore $R-X_t$ is the amount of radioactivity (counts/min.) remaining yet to enter the medium at time t.

RESULTS

^{24}Na *influx during steady states.* Time-courses for the movement of ^{24}Na into cerebral-cortex slices were obtained by incubating equal numbers of top and second-cut slices in glucose–bicarbonate medium containing ^{24}Na for gradated time-intervals. The total sodium exchanged varied greatly and consequently obscured any differences between stimulated and unstimulated slices. However, the average quantity of total sodium exchanged by unstimulated slices during the first four time-intervals correlated significantly ($r = +0·96$, $P < 0·01$, $n = 4$) with the average inulin space of these slices. An interval of exposure of approx. 90 sec. was required for a quantity of sodium in an individual slice equal to that calculated to be in the inulin space of that slice to come to the same specific activity as that of the medium. A highly significant correlation ($r = +0·88$, $P < 0·001$, $n = 20$) was obtained between sodium (μequiv./g.) exchanged and the sodium (μequiv./g.) of the inulin space in 20 slices, eight unstimulated and 12 stimulated, incubated for 90 sec. in ^{24}Na, whereas there was only a weak correlation between these parameters in a similar group of slices incubated for 120 sec. ($r = +0·34$, $P > 0·1$, $n = 21$). The average quantity of sodium in the inulin spaces of the first group of 20 slices was 54·3 μequiv./g., which, if equilibrated within 90 sec., would imply an average rate of equilibration with the inulin space of 2170 μequiv./g./hr.

These results suggest that the quantity of sodium exchanged with the non-inulin space can be calculated by subtracting from the total sodium exchanged the quantity estimated to be in the inulin space at the specific activity of the medium, as described by the equations under 'Expression of results: Influx' in the Experimental section. The corresponding time-courses for the average values of sodium exchange in the non-inulin space for the two steady-state conditions with and without stimulation are shown in Fig. 1. They are considerably less variable than time-courses based on the total sodium of the slice and indicate that the procedure used, although only approximate, is substantially justified.

Estimates of the 'initial' rates of sodium exchange in the non-inulin space from the initial slopes of the two curves drawn by eye in Fig. 1 give values of 175 μequiv./g./hr. for unstimulated slices and 1056 μequiv./g./hr. for stimulated slices. Presumably some quantity of isotope will exchange with sodium in the non-inulin space before the sodium of the inulin space is completely in equilibrium with the external medium, so that especially for stimulated slices the complex dynamic process occurring is not completely represented by the assumption implicit in the procedure, that exchange with the inulin space is complete before exchange with the non-inulin space begins. Nevertheless, these rates indicate that especially in unstimulated slices the equilibration of sodium is a much slower process in the non-inulin space than it is in the inulin space. Further, if all sodium is exchangeable, this unstimulated rate suggests a turnover of 10% of the sodium in the non-inulin space/min., whereas the larger quantity of sodium in the non-inulin space of stimulated slices (57·4 μequiv./g.) turns over about three times as fast (32%/min. or 3 mμequiv./g./impulse) as that in unstimulated slices (28·2 μequiv./g.).

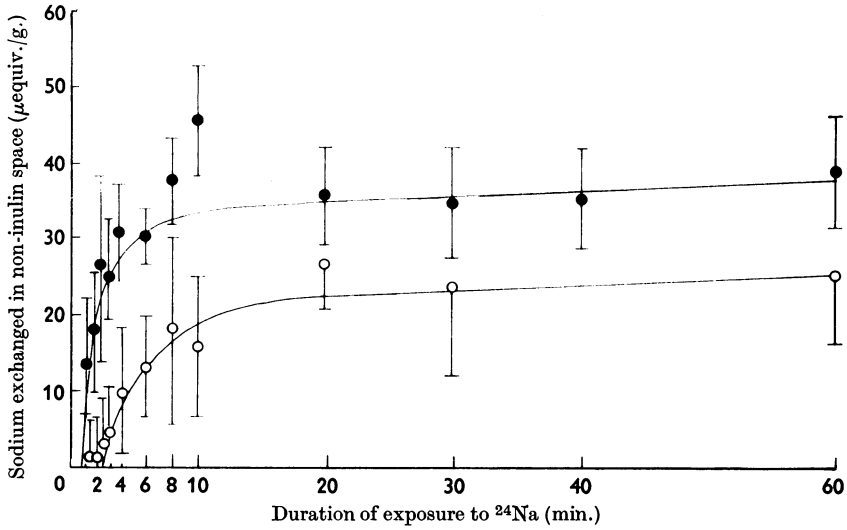

Fig. 1. Average ^{24}Na influx into the non-inulin space of cerebral-cortex slices, expressed as the quantity of sodium (μequiv./g. initial wt.) in the non-inulin space that has exchanged with sodium in the glucose–bicarbonate medium containing ^{24}Na tracer during periods of 1·5 to 60 min. ○, Unstimulated tissue; ●, tissue stimulated for 10 min. before the addition of ^{24}Na to the medium and throughout the interval examined. Each point represents the average of four to nine slices. Vertical bars indicate standard deviations.

After 60 min. exposure to medium containing ^{24}Na, complete or almost complete exchange with the sodium of the non-inulin space has occurred in both stimulated ($88 \pm 13\%$) and unstimulated ($93 \pm 9\%$) slices (98 and 97% respectively of the total sodium in the slice). Assuming therefore that at infinite time the specific activity of all the sodium in the non-inulin space would come to be indistinguishable from that of the medium, the quantity of sodium in the non-inulin space that has not exchanged during any particular time-interval was calculated for unstimulated and stimulated slices in the steady state. These results are plotted in Figs. 2 and 3 with semi-logarithmic co-ordinates.

Clearly sodium influx into the non-inulin space in either condition cannot be described by a single exponential process. However, by assuming two exponential components to be present in the results used for Figs. 2 and 3, a 'fast' one, which came to equilibrium in less than 20 min., and a 'slow' one, determined by the points from 20 min. onward, regressions for the two linear slopes were calculated by using logarithmic values (Hald, 1960). The 'slow' process calculated for unstimulated tissue did not differ statistically in either amount involved or rate from that for stimulated tissue (Table 1), and so the values of points from 20 min. onward for both were combined to give the resulting 'slow' slope, shown in Figs. 2 and 3, which differed significantly from zero ($t = 3{\cdot}43$, $P < 0{\cdot}002$, $n = 44$).

The resulting curves of Figs. 2 and 3 representing the sum of the 'fast' and 'slow' components appear to coincide reasonably well with the average values plotted for each time-interval except those at 8 and 10 min. Values at 8 and 10 min. for eight stimulated and 11 unstimulated slices (obtained in the same experiments) were eventually rejected in the final calculation on the basis that they deviated from the remaining portions of each curve comprised of about 80 slices, and when included in the present system produced results for the regression of the 'fast' slope that greatly underestimated the amounts of sodium in the non-inulin space.

By assuming that the two exponential processes derived above for the non-inulin space represent two compartments that do not communicate directly with one another and that are connected in parallel to the large constant reservoir of external medium and inulin space, each process may be regarded as independent, $Q_1 = C_1 e^{-\lambda_1 t}$ and $Q_2 = C_2 e^{-\lambda_2 t}$. Since each of these two-compartment open systems behaves mathematically as if there were no recycling of ^{24}Na (Solomon, 1960), then the rate constant, k, of transfer from each compartment into the reservoir may be identified directly with the respective exponential constant, λ, and the amount (μequiv./g.) of sodium involved in each process may be obtained from the respective zero intercept, C (Robertson, 1957). In the steady state the rate of transfer of sodium out of each compart-

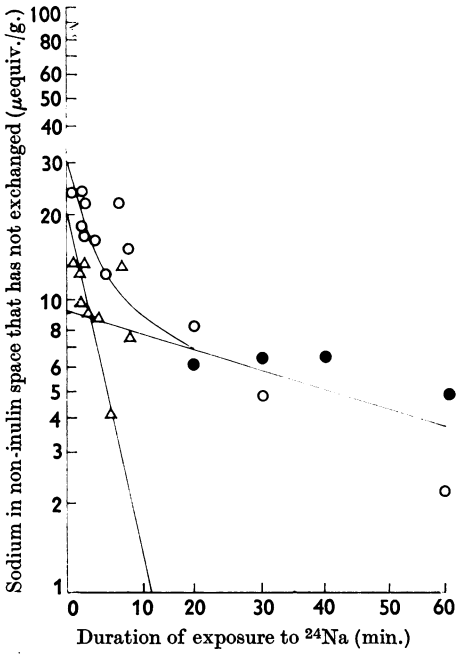

Fig. 2. Average quantity of sodium (μequiv./g.) in non-inulin space that has not exchanged during influx of ^{24}Na into unstimulated cerebral-cortex slices. ○, Experimental results for unstimulated tissue; ●, experimental results for stimulated tissue (plotted here only for time-periods longer than 10 min. duration). Regressions for a 'slow' linear component were calculated for the logarithmic values of all points (stimulated and unstimulated) from 20 min. onwards, and numerical values, obtained from the logarithms of points on this straight line less than 10 min. duration, were subtracted from results for individual slices at these time-intervals to give an estimate of the quantity of sodium involved in a 'fast' component. The average results of these calculations for each time-interval are represented on the graph by △. The regressions representing the 'fast' straight line were obtained from the logarithms of the individual subtractions. The curved line represents the sum of the 'fast' and 'slow' components.

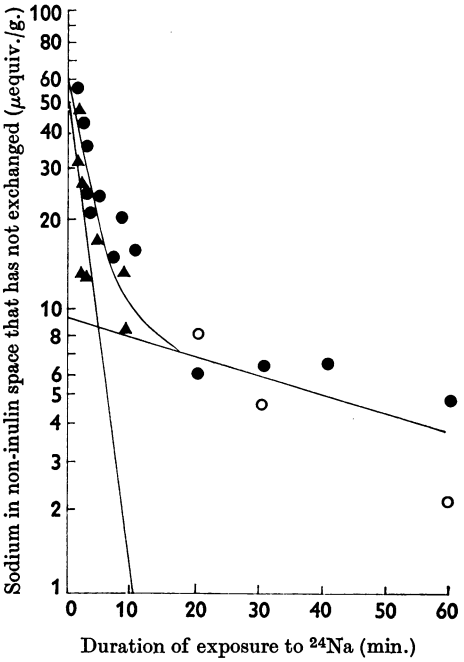

Fig. 3. Average quantity of sodium (μequiv. /g.) in non-inulin space that has not exchanged during influx of ^{24}Na into electrically stimulated cerebral-cortex slices in the steady state. ○, Experimental results for unstimulated tissue (plotted here only for time-periods longer than 10 min. duration); ●, experimental results for stimulated tissue. The straight lines, the curve and the calculated points (▲) were obtained in an analogous manner to those of Fig. 2.

ment equals the rate of transfer of sodium into that compartment, so that from the amount C (μequiv./g.) in a compartment and the rate constant, k (i.e. the fraction of that amount transferred out of the compartment per unit time), the rate of sodium turnover (μequiv./g./hr.) in each compartment during influx may be calculated for stimulated and unstimulated tissue.

The results of these calculations are summarized in Table 1. In such a system the quantity of sodium in the 'fast' component increased on stimulation by a factor 2·7 ($t = 13{\cdot}70$, $P < 0{\cdot}001$). The steepness of the slope also increased significantly ($t = 4{\cdot}04$, $P < 0{\cdot}001$) on stimulation by a factor 1·6. The result is that the estimate for total turnover increased by a factor 4·3 on stimulation. Keesey *et al.* (1965) demonstrated an increase by a factor 2·0 in the quantity of stable sodium in the non-inulin space (from 28·2 to 57·4 μequiv./g.). This increase of 29·2 μequiv./g. was the average of movements of sodium in both compartments and was therefore somewhat less than the increase of 32·9 μequiv./g. observed in the present work in the amount of the 'fast' component during stimulation.

If the two processes were arranged in series, the isotope flowing through the 'fast' compartment before entering the 'slow' one rather than in parallel as above, the differences would not be expected to be much more than the variation in the present results, considering the relatively slow slope of the second component compared with the first, even though the mathematics is much more complex (Huxley, 1960). The rate constants for the series case would be expected to be only slightly less, whereas the 'fast' component would be only slightly larger and the 'slow' component only slightly smaller than in the parallel arrangement.

Table 1. *Effect of electrical stimulation on exchange of sodium of the non-inulin space in steady-state conditions*

Data for and from the curves in Figs. 2 and 3 are summarized in this Table. Symbols: n is the no. of observations; α is the $\log_{10}$ value for the intercept C of each regression line with the ordinate; β is the $\log_{10}$ value for each slope λ; $t_{\frac{1}{2}}$ is the time in minutes for the regression line to fall to one-half of the initial value C. Turnover rates have been calculated in μequiv./g./hr. from the product of 60 (min.) times C times λ (min.$^{-1}$). Standard deviations are shown for α and β.

	'Slow' component			'Fast' component	
	Unstimulated	Stimulated	Combined	Unstimulated	Stimulated
n	19	25	44	40	38
α	1·0640±0·2675	0·8526±0·1250	0·9623±0·2100	1·2783±0·1125	1·7148±0·0962
β	0·0109±0·0070	0·0022±0·0033	0·0064±0·0019	0·1035±0·0341	0·1642±0·0320
C			9·1	19·0	51·9
λ			0·01476	0·2385	0·3784
$t_{\frac{1}{2}}$			47·0	2·90	1·83
Turnover rate			8·0	271·9	1178·3

Table 2. *Effect of electrical stimulation on exchange of sodium in the non-inulin space during the first 3 min. of exposure to* ^{24}Na *under four experimental conditions*

Sodium exchange is expressed as the average amount of sodium (μequiv./g.±S.D.; six slices for each category) at the specific activity of the medium that had entered and exchanged with sodium in the non-inulin space during each time-interval, and in parentheses the fraction (%) of sodium in the non-inulin space that had exchanged.

Duration of exposure to ^{24}Na (min.) ... Experimental condition	1·5	2·0	2·5	3·0
Initial response to stimulation	11·8±6·2 (27·8%)	14·6±10·8 (31·9%)	22·8±2·5 (50·5%)	29·5±3·1 (61·6%)
Steady-state response to stimulation	13·9±7·7 (23·5%)	17·6±8·0 (31·8%)	26·2±12·2 (49·2%)	26·0±6·5 (53·8%)
Recovery after cessation of stimulation	9·1±7·5 (17·9%)	16·0±12·8 (27·0%)	15·2±8·7 (28·8%)	15·0±10·0 (29·5%)
Unstimulated controls	0·98±5·2 (3·8%)	0·88±6·0 (3·7%)	2·9±5·9 (12·9%)	4·2±6·2 (18·4%)

Lacking independent knowledge of the relative sizes of the two processes if arranged in series, we did not pursue this possibility further.

^{24}Na *influx during non-steady states.* Besides the steady-state stimulated and unstimulated conditions described above, in which the quantity and concentration of total sodium remained constant throughout each experiment, the movement of radioactive sodium into cerebral-cortex slices was also studied during the first few minutes after turning on or off electrical stimulation. At these times the quantity and concentration of total sodium in the slice and in the non-inulin space were either increasing or decreasing, as described by Keesey *et al.* (1965). The behaviour of radioactive sodium in the non-inulin space over the first few minutes of each of these four conditions is summarized in Table 2, in terms of both the amount and the fraction of sodium exchanged during each time-interval.

In the present preparation no difference was obtained in the amount of sodium exchanged during the first few minutes between slices exposed to ^{24}Na during the initial response to stimulation and those exposed to ^{24}Na after stimulation had already been applied for 10 min. (Table 2). Since the concentrations of non-radioactive sodium in the non-inulin space were different in these two stimulated conditions, this result seems to indicate that the increased sodium exchange during electrical stimulation in the steady state, described in the previous section, may be attributed mainly to the effect of electrical stimulation rather than to differences in concentration gradients between stimulated and unstimulated slices.

During the first few minutes after electrical stimulation had ceased, radioactive sodium continued to enter the non-inulin space of these slices more rapidly than in unstimulated controls (Table 2), suggesting that the increased influx during

stimulation persisted and returned to the unstimulated value only gradually after termination of stimulation. Exposure of two slices to ^{24}Na during 10min. after cessation of electrical pulses resulted in exchange in the non-inulin space of 16·3 and 16·7 μequiv. of sodium/g. (55·7 and 56·7% respectively of total sodium in the non-inulin space) compared with $16·2 \pm 9·4$ μequiv. of sodium/g. (46%) after 10min. exposure to ^{24}Na in six unstimulated controls.

^{24}Na *efflux during steady states.* Movement of radioactive sodium out of cerebral-cortex slices incubated in steady-state conditions comparable with those under which most of the above measurements of ^{24}Na influx were obtained is shown in Fig. 4. The quantity of sodium in the non-inulin space of an average stimulated slice (53·5 μequiv./g.) in these efflux experiments was about twice that of an average unstimulated slice (26·0 μequiv./g.), so that to make stimulated and unstimulated conditions somewhat comparable the results in Fig. 4 have been expressed as the fraction of total transferred radioactivity, rather than the amount of radioactivity (counts/min.), that remained yet to enter the medium after a given time in non-radioactive medium. A greater average percentage of the total transferred radioactivity has moved out from stimulated slices than from unstimulated slices at each time-interval, the fractional increase in efflux from the total system on stimulation averaging $6·1 \pm 2·6$% for the eight time-intervals in Fig. 4. This is probably a lower limit, since transfer of radioactive fluid adhering to holders tended to contribute relatively more counts/min. to unstimulated than to stimulated efflux, whereas loss of radioactivity from tissue during rinsing tended to be greater from stimulated than from unstimulated slices. It is nevertheless several times larger than the average fractional increase obtained from a similar calculation based on the results of influx measurements for the total slice over a similar time-period. It would be advantageous to compare efflux from the non-inulin space components with the corresponding influx measurements in the non-inulin space, by using a similar approach to that used by Zadunaisky & Curran (1963) for ^{24}Na efflux from whole frog brain *in vitro*, but, since our system appears from influx measurements to be more complex than that for frog brain, to do so would require more information, with less variation, than it has been possible so far to obtain from mammalian brain slices, and so the agreement of results from efflux experiments with those from similar influx experiments is at present qualitative only.

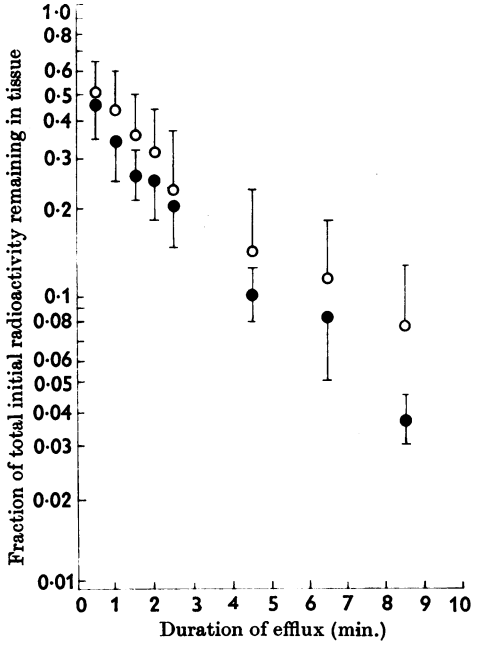

Fig. 4. ^{24}Na efflux from cerebral-cortex slices previously loaded with ^{24}Na, expressed as the fraction of the total initial radioactivity that remained in 100mg. of tissue at gradated intervals after transfer of the slices to non-radioactive medium. ○, Mean value at each time-interval for seven unstimulated slices; ●, mean value at each time-interval for three electrically stimulated slices in the steady state. The vertical bars indicate standard deviations.

DISCUSSION

Non-inulin space. The much more rapid diffusion of radioactive sodium into inulin than into non-inulin spaces of unstimulated cerebral-cortex slices described above, as well as the changes in ion content of the non-inulin space on electrical stimulation (Keesey *et al.* 1965), suggest that it is reasonable to use the non-inulin space as a measure of that portion of cerebral cortex that maintains ions at concentrations different from those of the external fluid and also maintains a negative potential with respect to the external fluid. However, the rates of ion movements described above should be regarded, along with the average measurements of membrane potential and of intracellular ion concentration, as characterizing electrically stimulated or unstimulated tissue as a whole rather than any particular cell type. The present results demonstrate a large increase, by a factor 4·3, in the turnover of the sodium of the non-inulin space in response to electrical stimulation. Measurements of ^{24}Na efflux are in qualitative agreement about the direction of the changes observed on electrical stimulation.

If the widely separate 'fast' and 'slow' rates obtained from Figs. 2 and 3 and Table 1 correctly reflect separate components in cerebral-cortex slices, the most likely arrangement would seem to be a parallel one, since if the compartments had been connected in series one would have expected that the 'slow' component would also increase on stimulation. This very slow but statistically significant component appears to have been independent of changes in the fast component, however, and so it most probably reflects uptake by connective tissue, vascular endothelium, or perhaps even a very slow increase in the damaged portion of the tissue such as the very slow increase in the size of the inulin space of brain slices found by Varon & McIlwain (1961) during prolonged incubation. Occurrence of a small proportion of weakly dissociated and slowly exchanging sodium is a further possibility.

Sodium entry. The average concentration of sodium in the non-inulin space of stimulated slices (85·3mM) was about twice that of the unstimulated slices (45·8mM), although both were still lower than the concentration of sodium in the external fluid (150mM). In similar slices the average membrane potential of unstimulated cells recorded intracellularly was about −60mV with respect to the external fluid (Hillman & McIlwain, 1961), whereas in brain slices stimulated electrically for 10min. in a manner similar to that used in the present experiments the cellular membrane potentials reached 'nearly zero' (Hillman *et al.* 1963). Entry of sodium into such slices may therefore be regarded as occurring down an electrochemical potential gradient in unstimulated tissue and down at least a small concentration gradient in stimulated tissue without the necessity of intervention by any transfer process requiring energy derived from metabolism.

However, at the same time that the average electrochemical potential was presumably decreasing, the average rate of sodium entry increased 4–6-fold on stimulation (Table 2) despite constant and identical external specific activities in the two conditions. The initial rate of entry of radioactive sodium into the non-inulin space during the first couple of minutes after applying electrical stimulation to slices was about as rapid, within the range of variability of the results, as that occurring during steady-state stimulation described above (Table 2), even though the concentration of sodium in the non-inulin space during this initial period of stimulation began at the unstimulated value of 45·8mM and continued to be 15–20mM less than the 85·3mM level obtained during prolonged steady-state stimulation. This result suggests that changes in membrane permeability determine the increased rate of sodium turnover immediately on applying electrical stimulation rather than possible increases in one-for-one sodium exchange (Ussing, 1947) due to increases in the sodium concentration in the non-inulin space.

Sodium extrusion. In comparison with a rate of about 1060 μequiv./g./hr. at which sodium entered the whole non-inulin space during the first few minutes after applying electrical stimulation, the amount of sodium in the non-inulin space of these slices rose during the same interval at the rate of about 460 μequiv./g./hr., achieving its steady-state level after approx. 6min. (Keesey *et al.* 1965). To account for this difference the rates of sodium extrusion must therefore have also increased on the onset of stimulation from the unstimulated rate of 175–275 μequiv./g./hr. to a rate of 600 μequiv./g./hr. (1060–460) during the first couple of minutes and finally after 6min. stimulation to the rate of 1060 μequiv./g./hr. observed during steady-state stimulation when, to maintain the constant content and concentration of sodium in the non-inulin space observed during stimulation for periods longer than 6min., the rate of sodium extrusion must have balanced the rate of sodium entry (Keesey *et al.* 1965).

The movement of sodium out of the non-inulin space of unstimulated brain slices is 'uphill' against both the electrical and chemical gradients mentioned above, and in stimulated slices it is at least against a concentration gradient of sodium between non-inulin space and external fluid. Such sodium extrusion requires a specific mechanism even if, as in 'exchange diffusion', no net change in sodium content is accomplished.

From ^{42}K measurements over much shorter intervals in slices cut without added fluid and incubated in media containing 2·8mM-calcium, Cummins & McIlwain (1961) obtained a rate of potassium entry of about 645 μequiv./g./hr. during the first few minutes of stimulation, which if applicable would conveniently balance the rate of sodium extrusion suggested to occur during this time-interval in the present group of slices. The similarity of rates may be only coincidental, however, and the presence of a one-to-one exchange with sodium rather than potassium to account for sodium extrusion has not been ruled out directly.

That at least part of the sodium exchange in brain slices requires energy derived from metabolism is suggested by the net extrusion of sodium first shown to occur in cerebral-cortex slices cut *in situ* without added fluid by Bachelard *et al.* (1962), who reported an initial rate of extrusion of sodium of 180–240 μequiv./g./hr. during the first 15–30min. of incubation and also after the termination of electrical stimulation. In neither case, however, was extrusion of sodium more than one-third to one-half complete. Keesey *et al.* (1965), employing modifica-

tions of this technique that were also used in the present study, obtained more complete recovery but at approximately the same rate of extrusion, 160 μequiv./g./hr. The rate of entry of sodium during the first few minutes after termination of electrical stimulation (Table 2) persists at an accelerated rate of approx. 450–960 μequiv./g./hr., closer to the stimulated than to the unstimulated condition. To effect the observed net extrusion of sodium at a rate of 160 μequiv./g./hr., the total rate of sodium extrusion during this time-interval must have been between approx. 610 and 1120 μequiv./g./hr.

Energy required for ion transport. By assuming that sodium and potassium movements are completely independent, the maximum amount of energy, W, required for these movements in cerebral-cortex slices in the steady state may be estimated from the equations (Ussing, 1949):

$$W_{Na} = RT \ln \frac{[Na]_e}{[Na]_i} + F(E_e - E_i)$$

$$\text{and } W_K = RT \ln \frac{[K]_i}{[K]_e} - F(E_e - E_i)$$

In the calculations the following values have been used: external sodium concentration $[Na]_e$, 150 mM; external potassium concentration $[K]_e$, 6·2 mM; average sodium and potassium concentrations in the non-inulin space, respectively 45·8 mM and 112·3 mM for unstimulated tissue and 85·3 mM and 69·6 mM for stimulated tissue; average membrane potential ($E_e - E_i$), -60 mV in unstimulated tissue and zero in maximally stimulated tissue as used in this investigation.

The maximum amount of energy required to transport 1 mole of sodium against the electrochemical gradient in unstimulated tissue is thus estimated as 2125 cal./mole, the corresponding value for potassium being 400 cal./mole. Sodium extrusion of 175–275 μequiv./g./hr. and potassium uptake of 330 μequiv./g./hr. (Cummins & McIlwain, 1961) imply maximal requirements of 0·5–0·7 cal./g./hr., assuming that no one-to-one sodium 'exchange diffusion' occurs. The corresponding estimates for stimulated tissue indicate that sodium transport requires only 350 cal./mole and potassium transport 1490 cal./mole. The maximum energy requirement for extrusion of 1060–1180 μequiv. of sodium/g./hr. and uptake of 645 μequiv. of potassium/g./hr. (Cummins & McIlwain, 1961) then becomes 1·33–1·37 cal./g./hr.

These calculations indicate that because of the depolarization during electrical stimulation less energy may be required to transport a mole of sodium than potassium in the maximally stimulated tissue, whereas in unstimulated tissue energy requirements for sodium extrusion exceed those for potassium uptake. Submaximally stimulated slices seem more likely to reflect the situation that ordinarily occurs *in vivo*.

Respiration is the major energy-yielding process in cerebral-cortex tissue, and if glycolysis is ignored will give minimal values for the energy available for ion transport. Twelve unstimulated cerebral-cortex slices in glycylglycine-buffered medium containing calcium at a concentration of 0·75 mM consumed oxygen at the average rate of 58 ± 4 μmoles/g./hr. (H. McIlwain, unpublished work). If a P/O ratio of 6 applies, 350 μmoles of 'energy-rich' phosphate/g./hr. would be available from respiration. If each 'energy-rich' phosphate bond provides 7600 cal./mole (Carpenter, 1960), then the total energy available from respiration in unstimulated tissue is 2·7 cal./hr., of which at most 26% is required for movements of sodium and potassium in the steady state. The increase in oxygen consumption on electrical stimulation of nine similarly incubated slices averaged 54 μmoles/g./hr. This would provide an additional 325 μmoles of 'energy-rich' phosphate/g./hr., corresponding to an additional 2·47 cal./g./hr. At most 35% (1·37 − 0·5 = 0·87 cal.) of this additional energy would be required for the additional ion movements observed in stimulated slices. These calculations would be invalidated if linked sodium–sodium or sodium–potassium exchange participates to some unknown extent in the rates obtained. Nevertheless, they suggest that a similar proportion of available energy is required for extrusion of sodium and uptake of potassium in both stimulated and unstimulated conditions.

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