

CONTINUOUS RECORDING OF CHANGES IN MEMBRANE
POTENTIAL IN MAMMALIAN CEREBRAL TISSUES
IN VITRO; RECOVERY AFTER DEPOLARIZATION
BY ADDED SUBSTANCES

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The membrane potentials which can be measured in isolated mammalian cerebral tissues, with micropipette electrodes, are modified by altering the composition of fluids which bathe the tissues, and by electrical stimulation. Hitherto, however, this knowledge has depended on measuring mean resting potentials from a number of cell elements, before and after the chemical or electrical change (Li & McIlwain, 1957; Hillman, Campbell & McIlwain, 1963). The cell potentials so obtained show appreciable variation: for example, guinea-pig cerebral cortex in a normal oxygenated glucose saline solution gave 59 ± 12 mV (Hillman & McIlwain, 1961). Measurement of small changes in potential, especially within brief periods, is therefore laborious or impracticable. Moreover, contribution is likely to be made to the spread of results by differences in the behaviour of different cell parts or types, and these merit individual examination.

Results are now reported with newly designed apparatus which enables an electrode to record from an individual cell of the isolated tissue for a period of some minutes, while the tissue is under satisfactory metabolic conditions and is exposed to successive solutions of different composition. The solutions have been applied to the tissue surface, a procedure which represents an *in vitro* counterpart to studies made of the brain *in situ* by applying solutions to its outer surfaces. Because of the present interest in applying substances in this way *in vivo* (cf. Feldberg, 1963), the time course of changes in potential following the application of two substances has been studied in detail and compared with the time course of their change in concentration calculated on the basis of diffusion. As added substances, potassium chloride and sodium glutamate were chosen. These are known to lower cerebral membrane potentials *in vitro* (Hillman & McIlwain, 1961). *In vivo*, glutamate and potassium chloride excite

cerebral neurones (Krnjević & Phillis, 1963) apparently by lowering membrane potential sufficiently to initiate spike potentials, as has been shown to occur in the spinal cord (Curtis, Phillis & Watkins, 1960). These substances have additional interest in relation to mechanisms of spreading cortical depression (Grafstein, 1956; Marshall, 1959; Ochs, 1962).

METHODS

Guinea-pigs were stunned by a blow on the back of the neck and exsanguinated by cutting the carotid arteries. For most experiments, slices of the cerebral cortex were cut rapidly, *in situ* and without added fluid, by the method of Hillman *et al.* (1963) and incubated in a Krebs-Ringer's glucose-bicarbonate saline solution (Hillman & McIlwain, 1961), which contained (mm): NaCl 124, KCl 5, KH_2PO_4 1.24, MgSO_4 1.3, CaCl_2 2.6, NaHCO_3 26 and glucose 10, and was equilibrated with gas mixture of 95% O_2 and 5% CO_2 . In some instances, slices were cut free-hand, using a thin strip of razor blade and without removing the brain from the body. The electrical measurements here reported refer to the first slice taken from the outer convexity of the cortex, usually from the fronto-parietal areas.

Apparatus

The apparatus was developed from that of Li & McIlwain (1957) and is shown in Figs. 1 and 2. The incubating medium in the slice-chamber *a* was maintained at 37° C by water in the thermostat bath *e*. The incoming gas (95% O_2 , 5% CO_2) had passed through a flow-meter and after passing through the sintered-glass bubbler it was moistened and warmed in its passage through the water in the thermostat bath, and then was guided across the moist surface of the tissue slice. This rested on a Nylon and Perspex grid *g*, and the gas passed out through the wide central opening above it. When the O_2/CO_2 gas mixture was supplied at 50 ml./min, gas collected 1 mm above the slice was found to contain 84% O_2 and 3.5% CO_2 .

The tissue was in immediate contact with about 8 ml. of medium. Additional fluid could be added from a syringe by way of a tube connected to one opening *b* in the slice-chamber. The other openings *b'*, *b'* were connected by fine tubing to a Watson-Marlow Flow Inducer, Type MHRV, enabling the incubating medium to be circulated at 1.25 ml./min. The thermostat bath was maintained by a heating coil and control system designed by Mr J. W. Sargent. The coil (*h*) consisted of 4 yd. of 29 s.w.g. Diamel-coated Cu-Ni wire. Initial rapid heating from cold was by means of a 24 V d.c. supply. When the temperature in the slice-chamber had reached about 35° C the control unit was switched on. This unit consisted of the thermistor *j* which controlled the current to the input of a transistorized d.c. amplifier, the output stage of which controlled the current through the heating coil. The circuit is such that as the medium approached 37° C the current through the coil decreased until at 37° C it was just sufficient to maintain the temperature. The unit's sensitivity can be adjusted so as to alter the rate at which it compensates for temperature changes. This method of maintaining temperature eliminates the electrical artifacts caused by switching larger currents suddenly, as in a conventional thermostat; there was also no need for a circulating pump or stirrer with attendant vibration.

Movements of chamber and micro-electrode. The Perspex and steel mounting (*k*, *k*) for the chamber formed a very rigid structure. The lathe slides (*l*, *l*) provided fine movement of the chamber system in a horizontal plane in two directions at right angles. Thus, it was only necessary to provide vertical movements for the recording micro-electrode. The vertical movement portion of a Prior micromanipulator was mounted on a horizontal brass bar supported at each end by the vertical brass pillars *v*, *v*. This was mechanically more stable than the previous arrangement with the micro-electrode at the end of an arm moving in

both vertical and horizontal planes. Vertical displacement of the micro-electrode was recorded on one channel of a pen-recorder by means of a potentiometer *p* driven by the fine control of the micromanipulator. Movement of $130\ \mu$ (one revolution of the fine control) gave a pen movement of 12 mm.

Micro-electrodes and micropipettes. The non-polarizable electrodes (*c* and *n*) consisted of AgCl-coated Ag wire sealed into a short length of glass tubing filled with 3% agar in saline solution. The micro-electrodes were glass micropipettes filled with 2.7 M-KCl. Tips were $< 0.5\ \mu$ and d.c. resistances were 10–50 M Ω .

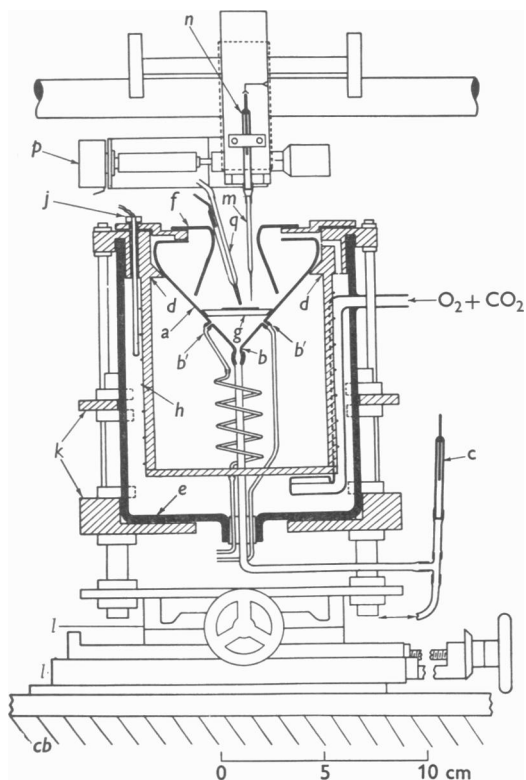


Fig. 1. Slice-chamber, thermostat, and electrode assembly. The conical glass slice-chamber *a* has openings *b*, *b'*; two for circulating fluid and one for the adjustment of fluid level and connexion to the indifferent electrode *c*. The chamber is supported by a Perspex frame *dd* which fits on top of a glass thermostat bath *e*. Baffle *f* deflects gas so as to pass over the moist surface of the tissue slice resting on grid *g*. The heating coil *h* is carried on a Perspex support and controlled by thermistor *j*. The Perspex and steel mounting for the chamber, *k*, is carried by the lathe slides *ll*, the lower of which is bolted to a steel plate fixed to the concrete block *cb*.

The micro-electrode assembly *n* carried a glass micro-electrode *m* flexibly attached to a non-polarizable electrode which plugs into a socket mounted on a Perspex block. This block carried an earthed shield and is attached to the face of a micromanipulator to give vertical movements which are signalled by the potentiometer *p*. The micro-injection pipette *q* is shown in position, but without its mounting, which is illustrated in Fig. 2.

The two non-polarizable electrodes were connected to a cathode-follower probe mounted close to the micromanipulator. Grid current was adjustable to $< 10^{-12}$ A. One side of the cathode follower was earthed to give single-sided operation. The probe formed part of a d.c. pre-amplifier, the output from which was fed to a cathode-ray oscilloscope and to one channel of the pen-recorder.

Fluids were applied to the surface of the tissue by a fine pipette, attached to an 'Agla' micrometer syringe (Fig. 2). The pipette was drawn by hand, from micro-electrode glass tubing, to a tip diameter of about 40μ , and sealed with picene wax on to a bent hypodermic tubing, to a tip diameter of about 40μ , and sealed with picene wax on to a bent hypodermic

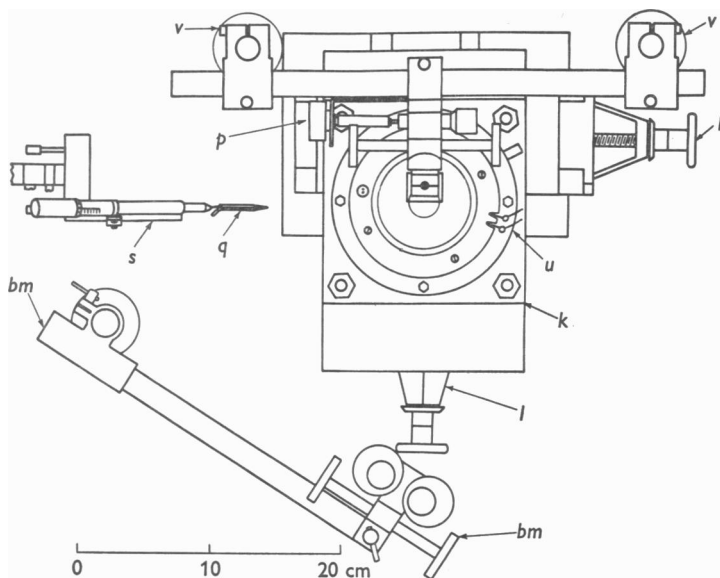


Fig. 2. Plan of the slice-chamber assembly on half the scale of Fig. 1. The micro-injection pipette *q* is shown withdrawn, mounted and fed by an Agla micrometer syringe *s*. The micro-electrode and associated apparatus shown more fully in Fig. 1 are supported on the vertical pillars *v*.

A binocular microscope and mounting, *bm*, can be swung into position over the slice. Connexions to the heater for the chamber are shown at *u*. Other lettering as in Fig. 1.

syringe needle. The micrometer syringe was fixed to a Prior micromanipulator mounted near the chamber. Double- and triple-barrelled pipettes were also made from similar tubing which was fused together before drawing. One barrel was fixed to the Agla syringe as described above, and the other barrel(s) attached by polyvinylchloride tubing to other micrometer syringe(s) placed near the chamber. Using the micrometer syringe it was possible to apply accurately as little as $0.2 \mu\text{l}$. of fluid to the tissue.

A binocular microscope *bm* was independently mounted on the base plate, and swung above the chamber when required, to view the tissue slice, pipette, and micro-electrode. An eye-piece graticule enabled horizontal distances between pipette and micro-electrode to be measured.

Procedure

The thermostat heating unit was switched on 45 min before the experiment commenced, to enable the slice-chamber to reach 37° C; gas flow was commenced at the same time. When the slice-chamber had reached 37° C, excess medium was poured into it and allowed to warm for 5 min. The fluid level was then adjusted to about 2 mm above the grid by the syringe connected to opening *b* (Fig. 1). Usually only one tissue slice was placed in the chamber and allowed to incubate in the medium for 30 min before electrical observations were commenced; a second jacketed chamber without electrodes and recording apparatus was used for maintaining other slices.

After the preliminary incubation, medium in the main apparatus was withdrawn by syringe until the tissue slice was just covered with fluid. Electrical observations were commenced by lowering the micro-electrode by the micromanipulator until it touched the surface of the medium above the slice. A reading of the micro-electrode position was then taken, and any small tip potential was backed off; electrodes with large tip potentials were rejected. The electrode was then lowered until it touched the surface of the tissue. At this point a brief change in potential usually occurred, and the micro-electrode position was again recorded, in order to measure the depth of fluid above the tissue. The electrode was then slowly advanced into the tissue until a negative deflexion appeared on the oscilloscope, when movement was stopped and the stability of the potential noted. A stable potential was taken as one which depolarized at a slower rate than 10 mV/min and which lasted for more than 1 min; in fact, most potentials were either much more or much less stable than this. Penetrations were repeated until a stable potential was discovered. When this occurred, the micro-injection pipette was lowered until its tip just touched the surface of the medium. The depth of fluid above the slice was usually about 100–200 μ , and the horizontal separation between pipette and electrode about 500 μ . It was easy to manoeuvre the pipette to within 200 μ of the electrode, but this often resulted in the penetrated cell being dislodged from the electrode when the pipette touched the fluid. It was undesirable to have the pipette in the medium until a stable potential had been recorded because of diffusion of the contents of the pipette barrels into the incubating medium.

The pipette was raised from the medium after fluid had been applied and potential changes observed; it was not replaced until a further region of stable potential had been discovered.

During penetrations, small changes in tip potential were often observed; large changes were usually associated with changes in resistance, indicating breakage or blockage of the electrode tip, in which case the electrode was replaced. From time to time between penetrations, at intervals of 10–15 min, medium was squirted from a Pasteur pipette over the surface of the slice to ensure that it remained wet. Experiments generally lasted about 2–3 hr.

Analysis of tissue

At the end of representative experiments, tissue slices (approx. wt. 50 mg) were removed from the chamber with a mounted, bent wire, drained by repeated contact with a glass slide, and weighed on a torsion balance. They were then ground in 4 ml. 6% trichloroacetic acid, and the mixture centrifuged after 30 min. Portions of the supernatant were analysed for Na and K, using an EEL flame photometer, and for Cl by Lowry's method (Lowry, Roberts, Leiner, Wu & Farr, 1954). In some experiments, the medium contained 1% inulin, and the inulin content of the tissue was determined, using portions of the extract made for analysis for ions above, by the method of Varon & McIlwain (1961). These estimations were carried out by Miss H. Scapeti. In certain instances, slices were weighed both before and after incubation; after 120 min, their weight had increased as indicated in Table 1, column (ii). Data for composition of incubated tissue are given in Table 1, column (i) gives data for the composition of fresh tissue.

Calculation of intracellular sodium and potassium involves assumptions about the composition of the extracellular fluid and the volume of the extracellular space. For fresh tissue, assuming the extracellular fluid to be of similar composition to the cerebrospinal fluid, extracellular ion concentrations can be calculated from the values given for guinea-pig plasma (Bachelard, Campbell & McIlwain, 1962) corrected by the distribution ratios for these ions between plasma and cerebrospinal fluid (Davson, 1960). The extracellular concentrations so calculated were: potassium, 3 mM; sodium, 151 mM, and Cl, 123 mM. The estimates in columns *i(a)* and *i(b)* were then derived from chloride values, but on the basis of two different assumptions. In *i(a)* the chloride is considered to be in electrochemical equilibrium with observed (Phillips, 1956) cortical membrane potentials of -60 mV. This afforded a ratio $[Cl]_e/[Cl]_i$ of 9.3, and thus $[Cl]_i = 13$ mM. Eccles (1957) made a similar assumption for motoneurons but on the basis of a membrane potential of -70 mV, so obtaining a $[Cl]_i$ of 9 mM. In *i(b)* chloride is assumed to be distributed in the ratio observed by Keynes (1963) to obtain at the squid axon. Then $[Cl]_e/[Cl]_i = 4.6$, and $[Cl]_i = 27$ mM.

TABLE 1. Ion composition of guinea-pig cerebral cortex. Values for observed data give the mean results \pm S.D., with the numbers of observations in parentheses. Those in column (ii) are for tissue sampled immediately after measurement of membrane potentials. For details of calculations, see text; those giving values of column (ii) were made on the basis of the final weight of tissue after incubation. Suffix *e*: external and *i*: internal.

Constituent	(i)		(ii)	
	Fresh tissue		Incubated tissue	
Observed				
Increase in weight (mg/g)	—		254 \pm 55 (9)	
Na (μ -equiv/g)	63 \pm 5 (20)		101 \pm 7 (24)	
K (μ -equiv/g)	103 \pm 3 (20)		70 \pm 5 (26)	
Cl (μ -equiv/g)	43 \pm 7 (14)		82 \pm 9 (25)	
Inulin space (μ l./g fresh wt.)	—		490 \pm 60 (18)	
Calculated				
	(a)	(b)	(a)	(b)
Intracellular Cl (mM), Cl_i	13	27	14	—
Cl of extracellular space (μ -equiv)	30	16	68	—
Extracellular space (μ l./g) based on Cl distribution	244	130	515	—
Cl of inulin space (μ -equiv)	—	—	—	65
Cl of non-inulin space (mM), Cl_i	—	—	—	33
Ratio Cl_e/Cl_i	9.3	4.6	9.3	4.0
K of extracellular space (μ -equiv)	0.7	0.4	3.2	—
Intracellular K (mM), K_i	135	118	136	—
K of inulin space (μ -equiv)	—	—	—	3.1
K of non-inulin space (mM), K_i	—	—	—	131
Ratio K_i/K_e	45	39	22	21
Na of extracellular space (μ -equiv)	37	20	78	—
Intracellular Na (mM), Na_i	35	49	47	—
Na of inulin space (μ -equiv)	—	—	—	74
Na of non-inulin space (mM), Na_i	—	—	—	53
Ratio of Na_i/Na_e	0.23	0.32	0.31	0.35

Values for the incubated tissue can also be treated according to assumptions *i(a)* and *i(b)*, and column *ii(a)* gives the results of calculations based in the same fashion as *i(a)*, i.e. on chloride distribution according to observed membrane potential, $[Cl]_e/[Cl]_i$ again being 9.3. This calculation ignores the inulin measurements. When, in the incubated tissue, it is assumed that the inulin and non-inulin spaces correspond to the extracellular and intracellular spaces of the tissue, values for the composition of incubating medium and tissue enable the calculation of intracellular ion concentrations to be made, with the results quoted in column *ii(b)*.

RESULTS

Potentials and tissue composition

Micro-electrodes on penetrating slices normally registered abrupt negative potentials, of -40 to -80 mV. In 2000 penetrations, 113 stable potentials were recorded; many potentials were observed which did not fulfil the conditions of stability as defined under Procedure. Examples recorded by pen-writer are shown in Fig. 3, that of trace *A* being typical.

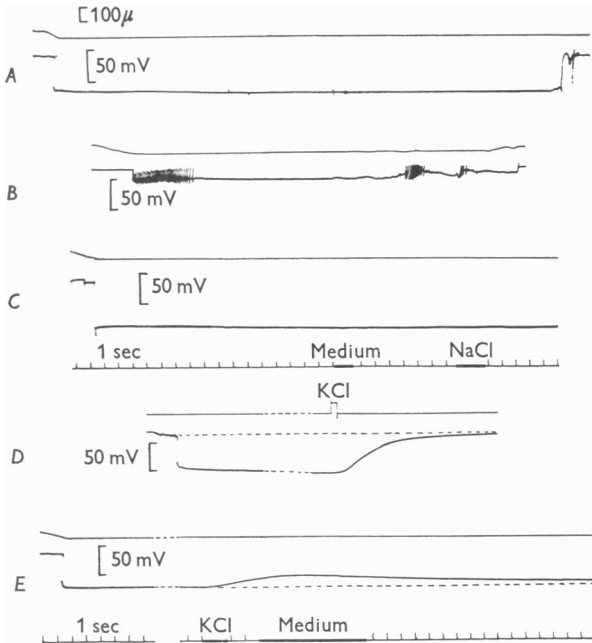


Fig. 3. Potential changes on penetrating a slice from the parietal region of the guinea-pig cerebral cortex, maintained *in vitro*. Records by pen-recorder on paper moving at 0.75 cm/sec. In *A*, *B*, *C* and *E* upper line records movements of the electrode and the lower line the potential observed. In *D* the upper line records only the addition of KCl. In each case the micro-electrode had moved by various amounts before the record commenced; thus the upper line does not show the total distance travelled by the electrode in the tissue.

A. Region encountered at a penetration of 100μ , of -53 to -57 mV, and lasting 35 sec.

B. Region at a depth of 130μ , with entry accompanied by spike discharge.

C. Region at a depth of 150μ , of -68 mV. An addition of 1.0μ l. incubating medium after 17 sec was followed by an addition of 1.0μ l. 0.5 M-NaCl 8 sec later.

D. Region of -59 mV initially, becoming -69 mV over a period of 20 sec before the addition of 0.32μ l. of 2 M-KCl.

E. Region at a depth of 80μ , of -56 mV. An addition of 1.0μ l. 0.5 M-KCl after 30 sec was followed by an addition of 5μ l. incubating medium 2 sec later. Note that 1 sec markings have not reproduced well in the Fig. and that the line in *E* below 1 sec is of length corresponding to 8 sec.

As indicated by the upper line, movement of the electrode was halted when the negative potential was registered, and the value remained between -53 and -59 mV during 35 sec, after which it abruptly fell, becoming zero after some brief irregularities. The beginning of the record of electrode movement is not shown in this Figure; the electrode was, in fact, halted at a depth of 100μ . For stable potentials, the mean and s.d. of the maximum values registered on the 113 occasions was 59 ± 10 mV. Figure 4 gives the distribution of durations of these potentials. There was no correlation between depth in slice and size or duration of potential.

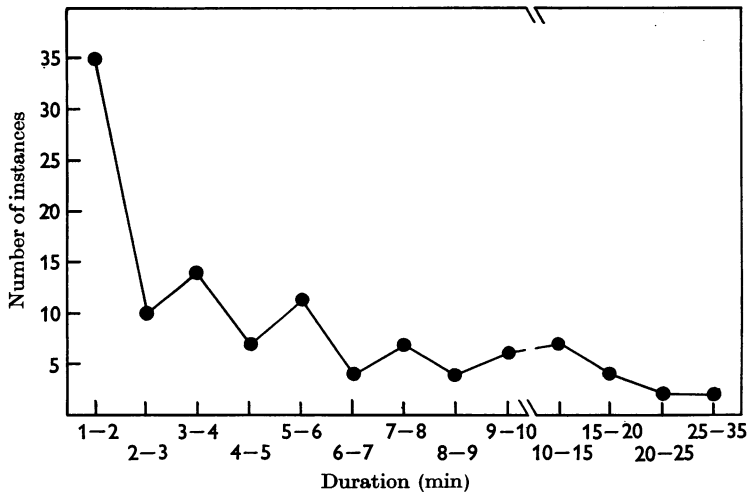


Fig. 4. Persistence of the 113 potentials observed in guinea-pig cerebral cortex, which were stable in the sense that they lasted for at least 1 min and declined at less than 10 mV/min. Each point represents the number of potentials which became unstable during the interval stated as abscissa.

Occasionally, spike discharges were recorded on entering a cell, or subsequently; an example is recorded in Fig. 3B observed at a depth of 130μ . The frequency response of the pen-writer was too low to record accurately the shape of the spikes, but changes can be seen in frequency of discharge in the first burst of spikes: this and other characteristics of the discharges remaining within the description given by Li & McIlwain (1957) and by Hillman *et al.* (1963). Tissue samples were removed from the apparatus after incubation in normal media and after the micro-electrode penetrations which had afforded the data of Fig. 4, and were extracted for determination of sodium, potassium and chlorine. Comparison of the values obtained with those for the cerebral cortex before incubation is given in Table 1. Considerable gradients of each ion are maintained

TABLE 2. Duration and amplitude of potentials recorded in tissues by different cutting methods and incubated in media differing in calcium content

The experiments were performed in the order A-D listed in the first two columns. The 34 potentials of Group A are included in Fig. 4.

Group	Cutting method	Ca of media (mm)	Number of cells having potentials lasting:										Total cells	Mean potential (\pm s.d.) (mV)		
			1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	Over 10				
A	With guide	2.6	12	3	4	2	3	1	2	1	2	1	2	4	34	58 \pm 10
B	With guide	0.75	11	9	5	0	2	1	1	1	0	2	2	1	32	57 \pm 8
C	Freehand	0.75	14	5	1	5	1	0	0	2	2	2	2	2	32	54 \pm 7
D	Freehand	2.6	7	2	3	2	4	3	4	1	1	0	1	1	27	59 \pm 10

Comparison of cutting methods

Group	Less than 4 min	Over 4 min	Significance level
B	25	7	$P = > 0.50$
C	20	12	
D	12	15	$P = 0.80$
A	19	15	

Comparison of Ca levels

Group	Less than 4 min	Over 4 min	Significance level
B + C	45	19	$P = 0.025$
D + A	31	30	

between tissue and incubating fluid, though the gradients are less steep than in the original tissue (see Discussion).

Altering calcium, potassium and cutting method. In the experiments just reported tissue was prepared *in situ*, using a blade and guide as described by Hillman *et al.* (1963), and incubated in a medium containing 2.6 mM calcium salts. Further experiments were performed in order to examine jointly the effects of (a) minimizing manipulation by cutting tissue slices free-hand without the use of a guide, and (b) using media containing 0.75 mM calcium salts, this approximating to the free rather than the total calcium content of blood plasma. No difference in potential was produced by the different cutting methods (Table 2). On grouping results so that the incidence of potentials lasting less than 4 min was compared with the incidence of those lasting more than 4 min, the potentials recorded in media 2.6 mM in calcium were found to be more stable than those in the media lower in calcium. Media 2.6 mM in calcium were used in subsequent experiments. In three experiments, the incubating medium contained 3 mM potassium and 0.75 mM-Ca. After 2 hr incubation the tissue contained 49 μ -equiv/g K (compared with 70 μ -equiv/g in tissue incubated with 6.2 mM-K, see Table 1). Only three stable potentials were obtained, of average amplitude 47 mV and duration 4 min. It is difficult to draw firm conclusions from such a small sample, but the potentials are definitely smaller than those of group C (Table 2), and the incidence of stable potentials in these experiments was less than half that of comparable experiments with 6.2 mM-K.

Surface application of medium and NaCl. Additions of a few μ l. of media or of NaCl solution were made to the surface of the slice, immediately above the region penetrated by the micro-electrode. Such additions were without effect on the potentials recorded. Figure 3C records such an experiment: again, movement of the electrode (upper trace) was stopped when the steady potential of -68 mV was recorded. After 17 sec, 1 μ l. of medium was added without causing change in potential, and was followed at 25 sec by a similar volume of 0.5 M-NaCl. Despite its hypertonicity this also caused no change in potential; the region being observed was approx. 150 μ below the surface of the tissue to which the additions were made. In subsequent experiments, addition of medium was frequently made as a control before the application of other agents.

Displacement of potential by added KCl

Membrane potentials were previously found to be lost when cerebral cortical tissues were bathed in solutions of K content above 20-30 mM (Hillman & McIlwain, 1961). The experimental arrangements described in the Methods section now enabled quantities of 0.1-2 μ l. of K-rich

solutions to be applied by micrometer syringes to the tissue surface, close to a micro-electrode and while the electrode was giving continuous potential readings. On adding 0.5–2 M-KCl in this way, a prompt change in potential was seen (Fig. 3, *D* and *E*; Fig. 5). Within 1–2 sec of commencing the addition, the potential fell and in 5–10 sec could reach complete depolarization. In other instances, when depolarization was not complete, the minimum potential reached was retained only momentarily, and gradual recovery commenced in 15–60 sec (Fig. 3*E*).

The behaviour of the applied fluid was studied by filling a syringe barrel with methylene blue solution. On application, fluid spread rapidly in a roughly circular patch, into the thin layer of medium which remained above the tissue slice. The obvious spread of this patch was complete at the moment the micrometer of the pipette stopped turning: that is, there was no noticeable time-lag between movement of micrometer and movement of fluid. During its spread, the methylene blue engulfed the shank of the micro-electrode; the area covered by the stain depended on the volume applied and the depth of fluid above the slice. The rapid spread of methylene blue during application was followed by a slow diffusion out into the incubating medium, and down into the slice.

Under these conditions, an approximate measure of the concentration of K^+ in the vicinity of the cell element under observation should be obtainable by considering the applied KCl as forming a plane source, the cell being situated at a point at a variable distance from the source. The concentration at the cell is given by the following equation (Jost, 1960, p. 23)

$$C = \frac{C_0}{2} \left[\operatorname{erf} \left(\frac{h+x}{2\sqrt{Dt}} \right) + \operatorname{erf} \left(\frac{h-x}{2\sqrt{Dt}} \right) \right] \quad (i)$$

where C = concentration at the cell; C_0 = concentration of KCl applied at surface; h = depth of fluid above the slice; x = vertical distance from the surface of the fluid to the cell under observation; D = diffusion coefficient for K^+ , taken as $2.5 \times 10^{-5} \text{ cm}^2 \text{ sec}^{-1}$ in free solution at 37° C ; t = time, and erf = the error function.

This equation is approximate only, since it treats the portion of tissue containing the cell as a vertical cylinder. In practice, as the KCl diffuses down into the slice, it is also diffusing across the walls of the 'cylinder' and thus increasing its volume. Therefore the concentration of K^+ at the cell will be less than that calculated from the equation by an amount depending on the depth of the cell in the slice. A rough correction factor is given by

$$\frac{r^2}{(r+x)^2}$$

where r = the radius of the circle of tissue surface covered by the applied KCl solution. This is 1700μ for an addition of $1 \mu\text{l.}$, and afforded a correction factor of 0.74 where $x = 300 \mu$.

Alternative formulations which were considered treated the applied KCl: (*a*) as a point source, and (*b*) as a plane source, but neglecting the thickness of fluid h . Assumption (*a*) gave values of K concentration far in excess of those possible, while (*b*) gave very different potential/concentration time relations. Questions regarding the extracellular space through which diffusion is presumed to occur are considered in the Discussion.

The points and the right-hand scales of Fig. 5 show the time course of the observed change in potential following the addition of $1 \mu\text{l.}$ of 0.3 or 0.5 M-KCl to slices in which an electrode had encountered cells at depths of 140–200 μ . The resting potentials of the cells were similar before the additions were made, but diminished in one case about 20 and in the other

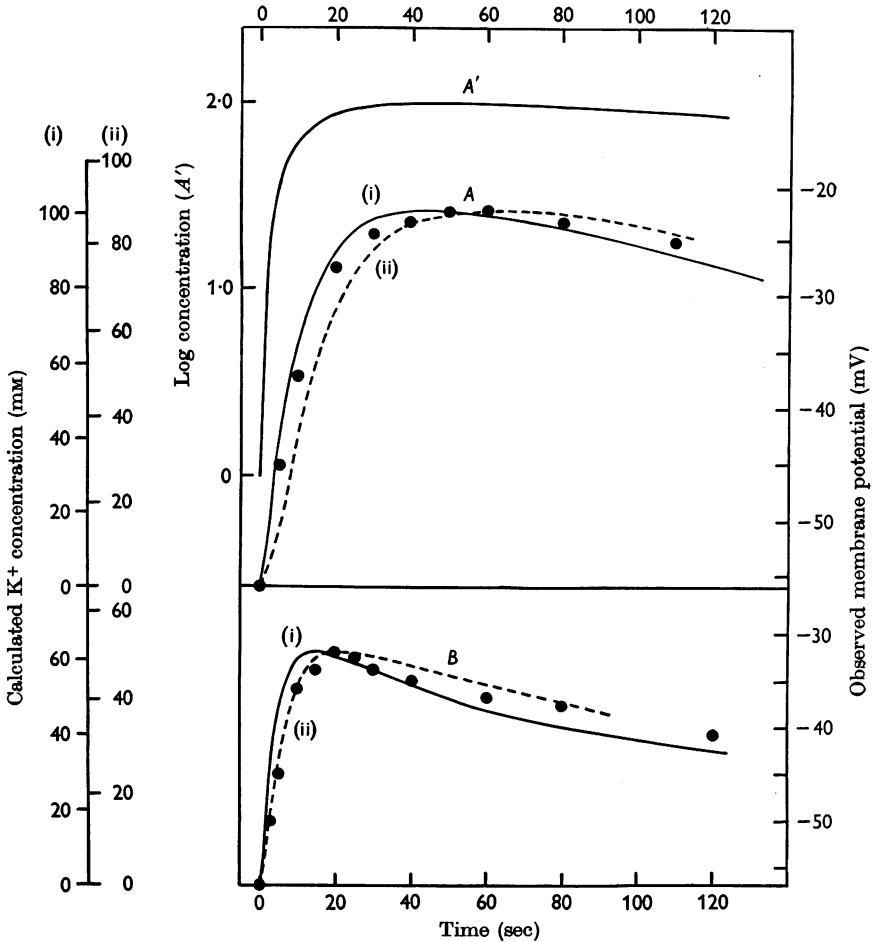


Fig. 5. Observed change in resting potential (shown by points) following a small addition of KCl to the tissue surface; comparison with the time course calculated for change in K^+ concentration (from eqn. (i) solid lines, scale (i) on left; with increased diffusion-path correction interrupted lines, scale (ii) on left). The cell *A* of which the potential is represented by the points near curve *A* had a resting potential of -56 mV and was 200μ deep in the tissue slice, which was covered to a depth of 300μ by incubating medium. One microlitre of 0.5 M-KCl was added at zero time, and the concentration of KCl at the cell was calculated for various times after the addition, using the formula given in the text. Above (*A'*) are shown the logarithms of the calculated potassium concentrations.

In curve *B* the cell had a resting potential of -57 mV and was 140μ deep in the tissue slice, which was covered to a depth of 150μ by incubating medium. One microlitre of 0.3 M-KCl was added at zero time, and the concentration of KCl at the cell, *B*, calculated as above.

The lines are drawn freehand through a series of calculated concentration/time values.

by about 35 mV. After 15 sec in one case and 50 sec in the other, repolarization commenced, and proceeded throughout the remainder of the 5–6 min of observation. Also plotted in Fig. 5 (solid lines and left-hand scales (i)) are the external K concentrations calculated from the above equation (for lines and scales (ii) see Discussion). The observed potentials are suitably scaled so that in each case their maxima are at the same height on the ordinate as the calculated maximal concentration. It is then seen that the curves are of the same general form, but that the observed maximal

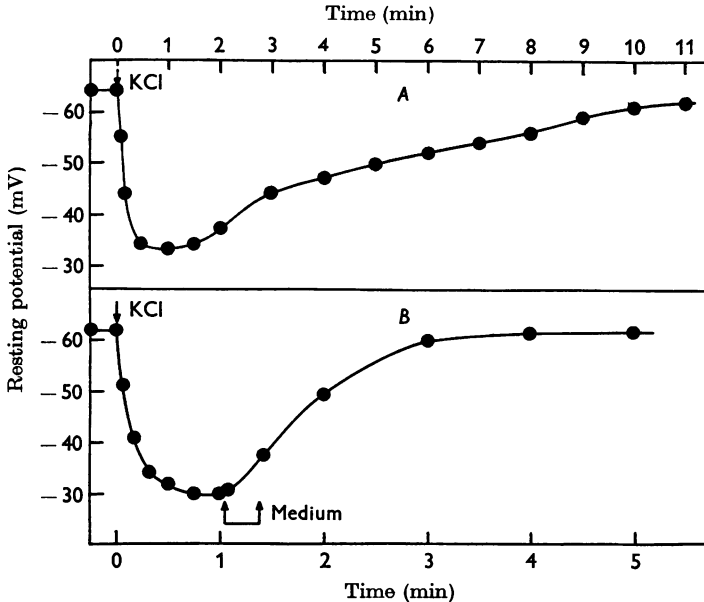


Fig. 6. Observed change in resting potential following a small addition of KCl to the tissue surface; showing also the effect of added medium on the time course of recovery. In *A* the cell was $150\ \mu$ deep in the tissue slice and $1.0\ \mu\text{l.}$ of $0.5\ \text{M-KCl}$ was added 1 min after penetration, shown as zero on the abscissa. In *B*, $1.0\ \mu\text{l.}$ of $0.5\ \text{M-KCl}$ was added to the same cell 2 min after the end of *A*, followed 1 min later by $11.2\ \mu\text{l.}$ medium.

depolarization takes place after the calculated maximal concentration has been reached. The divergence is considered more fully in the Discussion. Agreement between the course of change in potential and that in the logarithm of the calculated concentration (curve *A'*, Fig. 5) is appreciably less satisfactory.

The return of membrane potential to the resting level was found to be accelerated by the application of medium at varying time intervals after the addition of KCl. Instances are shown in Fig. 6, repolarization being complete some 3–4 min after addition of KCl, whereas without washing

(Fig. 6A) recovery was only partial at 6 min. Also, a saline wash under favourable conditions allowed more than one addition of KCl to be made to a single cell, with recovery of potential to the resting level in between additions.

Displacement of potential by glutamate

Addition of small volumes of 0.1–0.5 M-sodium L-glutamate to the surface of the tissue in the fashion just described also resulted in prompt diminution of membrane potential at underlying cell-elements (Fig. 7). In all of twenty-two instances when glutamate was first added to such tissue, potentials became 10–30 mV less negative in 30–60 sec. This change was similar to or a little greater than that of some 14 mV, pre-

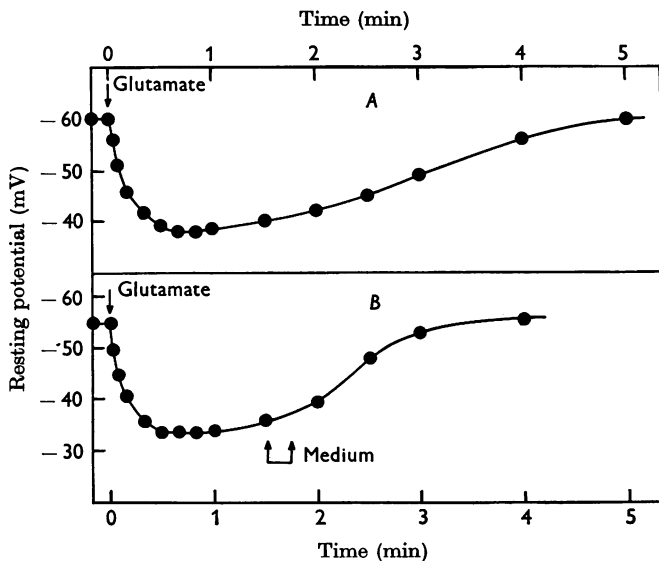


Fig. 7. Observed change in resting potential following a small addition of sodium glutamate to the tissue surface; showing also the effect of added medium on the time course of recovery. In *A*, the cell was 150 μ deep in the tissue, and 1.0 μ l. of 0.2 M-glutamate was added after 10 sec. In *B*, the cell was 180 μ deep, and addition of 1.0 μ l. of 0.2 M-glutamate after 10 sec was followed by 10 μ l. medium 90 sec later.

viously observed when the whole of the saline solution bathing isolated cerebral cortex was replaced by a medium 20 mM in L-glutamate (Hillman & McIlwain, 1961). In one instance, the glutamate concentration expected to be reached by diffusion was calculated, at the depth in the slice from which the micro-electrode was recording. The calculation followed the method of the preceding paragraph, adopting a diffusion constant for glutamate of $1.1 \times 10^{-5} \text{ cm}^2 \text{ sec}^{-1}$ (Longworth, 1953), with the result indicated in Fig. 8. The maximum glutamate concentration computed at

the electrode, and the minimum potential difference, were reached 40 sec after addition of glutamate commenced; thereafter, the concentration slowly declined and the potential slowly returned towards its previous value. Return of potential to its resting value was accelerated by washing with saline solution, as described in the preceding section; a typical example is shown in Fig. 7 B.

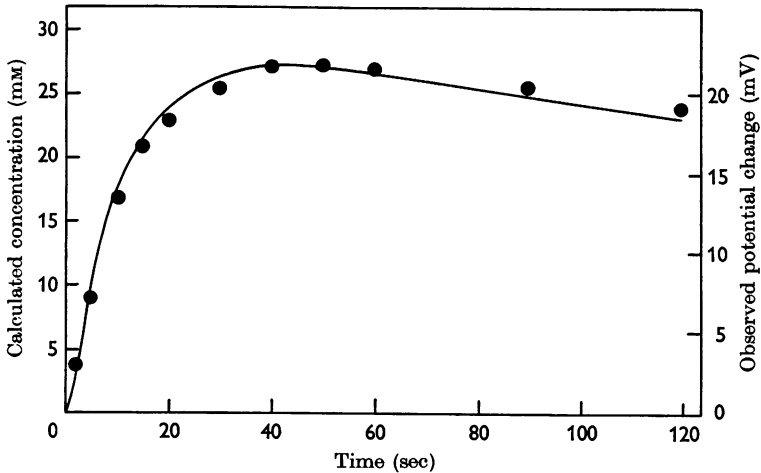


Fig. 8. Observed change in resting potential (shown by points) following a small addition of sodium glutamate to the tissue surface; comparison with the time course calculated for change in glutamate concentration (represented by line). The resting potential was -59 mV, which is shown as zero on the right-hand scale. The cell was 80μ deep in the tissue slice, which was covered to a depth of 120μ by incubating medium. One microlitre of 0.2 M-glutamate was added at zero time and the time course of glutamate concentration at the cell was calculated from the formula given in the text.

In twelve experiments in which 0.1 M-glutamate was applied, the calculated initial concentration of glutamate to give just observable depolarization, of 3 mV, was 0.2 mM. This compares closely with a threshold value for stimulation of cortical neurones *in vivo* of 0.14 mM calculated by Krnjević & Phillis (1963). In one particular cell, five successive additions of 1μ l. of 0.1 M-glutamate were made during 15 min and produced changes in potential ranging from 12 to 16 mV, with recovery to the resting level of 60 mV (aided by saline washes) in each case. Finally, an addition of 0.5 M-glutamate produced a change in potential of 24 mV followed by partial recovery and then a gradual depolarization to zero. The 0.1 M-glutamate was calculated to produce peak concentrations at the cell of about 10 mM.

It was noticed that when the concentration of applied glutamate and the depth of cell in the tissue were such that the peak glutamate concentra-

tion reached 20–30 mM, successive additions of glutamate produced progressively smaller depolarizations. In one cell, three additions of 0.2 M-glutamate during 15 min, giving peak concentrations of about 30 mM, produced successive depolarizations of 20, 15, and 10 mV, with complete recovery in between each addition and after the final one. The above cycle of additions was completed on two further occasions; other cells spontaneously depolarized after one or two additions.

When higher peak concentrations of glutamate were reached, cells appeared to become unstable and to depolarize progressively to zero, though usually with a partial recovery from the glutamate depolarization before the steady depolarization set in. The glutamate levels necessary for this to occur were around 50 mM.

DISCUSSION

The sodium, potassium and membrane potentials of normal cerebral tissue

The conclusions drawn about cell composition from the data of Table 1 assume the cellular phase of the tissue to be uniform in its ion relations with extracellular fluids. This can be a working hypothesis only, but the micro-electrode measurements of potential give, from highly localized regions, information which depends on the ion relations and suggests a relative uniformity. The regions of negative potential encountered by the electrode tip occupied about 30 % of the tissue volume (Hillman & McIlwain, 1961), or (see below) some 50 % of its cellular volume. Of these regions, about 10 % form the subject of the present observations, this selection having been made in order to obtain potentials stable for 1 min or more. The mean potential of the regions now examined, $-59 \text{ mV} \pm 10$, is close to the value of $-59 \text{ mV} \pm 12$ of the previous study and also to that of -60 mV observed by Phillips (1956) in Betz cells of the motor cortex of the cat, *in situ*. The incidence of potential values found by Hillman & McIlwain (1961) gave no indication of bimodal or more complex distribution, nor was the magnitude of the present potentials correlated with their location in the slice or with their persistence.

Comparison of numerical data for tissue composition and membrane potential is thus merited. The observed potentials of -60 mV are markedly less negative than the potassium equilibrium potentials corresponding to the observed ratios $[K]_i/[K]_e$ of 21 to 45 (Table 1), which are -83 to -102 mV . The simplest further assumption in considering tissue which is in a steady state *in vivo* or *in vitro* is to invoke the opposing sodium gradient, with the Nernst equation in the form (Hodgkin, 1958)

$$V = \frac{RT}{F} \ln \frac{[K]_e + b[Na]_e}{[K]_i + b[Na]_i}, \quad (\text{ii})$$

b giving the apparent permeability of Na relative to that of K. At 37° C, giving constants their usual values and taking activities as proportional to concentrations, it was found with the data of Table 1 that b was required to be 0.07–0.08 in the fresh tissue or 0.06 in the incubated tissue to give values of –60 mV. It has been concluded with the giant axons from squids in their resting condition, and also in muscle, that b is close to 0.01 (Hodgkin, 1958); in perfused squid axon, the corresponding value was 0.08 (Baker, Hodgkin & Shaw, 1962). On excitation in the squid it can rise momentarily to 20 (Hodgkin & Katz, 1949). The values for b necessitated by the present data thus come within the range observed in the resting systems.

Tissue fluids

When the incubated tissues were taken for analysis at the end of the present experiments they were drained from the fluid in which they had been immersed, but drained briefly only, in order to avoid loss of intracellular constituents. This procedure is known to leave fluid adhering to the tissue, and so to contribute to the variability of results noted in Table 1; Varon & McIlwain (1961) concluded that under these conditions 85–110 mg of adhering fluid remained per g of tissue. The fluid imbibed by the tissue was thus 254 – 98, or 156 mg/g. Taking tissue and fluid densities as 1 and the swelling as uniformly distributed, the increase in linear dimensions implied by this value is 5%.

The quantity of inulin associated with 1 g of incubated tissue was much greater than that contained in 254 μ l. of incubation medium; presumably 490 – 254 = 236 μ l./g of fluid in the fresh tissue, as sampled in the present experiments, is accessible to inulin. This merits comparison with the spaces in which the chloride of the fresh tissue may be distributed, and two calculations are quoted in Table 1 (see Methods section). In *i(a)* Table 1, the tissue chloride is assumed to be distributed passively according to observed (Phillips, 1956) membrane potentials of –60 mV, that is, that

$$\frac{RT}{F} \ln \frac{[\text{Cl}]_i}{[\text{Cl}]_e} = V \quad (\text{iii})$$

in addition to relation (ii). Extracellular space based on this chloride distribution is then 244 μ l./g tissue, in good agreement with the 236 μ l./g based on inulin and fluid uptake. This value has been adopted as most probably correct. It may be noted that if chloride is assumed to be excluded from the cellular phase, or in equilibrium with a more negative membrane potential, extracellular space based on chloride becomes greater, as suggested by Pappius & Elliott (1956). If chloride is supposed to be actively assimilated to cerebral tissues as to the squid giant axon

(Keynes, 1963), the apparent extracellular space is smaller, e.g. the 130 $\mu\text{l./g}$ quoted in Table 1, i(b). These results are only partly relevant to the divergent conclusions about extracellular space in the cerebral cortex *in situ* (see Van Harreveld & Schadé, 1960; Katzman, 1961; Ochs, 1962; Eccles, 1964) for certain movements of fluid and electrolyte may already have occurred in the tissue as sampled in the present experiments. They suggest, however, that the incubated tissue contains 0.392/1.156 or 34 % of extracellular space.

Addition of KCl

It is satisfactory that the present system demonstrates depolarization by added KCl and subsequent recovery, with a time course which can be accounted for to the degree indicated in Fig. 5. Divergences from the quantitative expectations of Fig. 5 are also in general understandable.

Thus, considering first the maximal change in potential; this is less than the values obtained by substituting the maximal calculated $[\text{K}]_e$ in eqn. (ii), with the appropriate b values. Cell A had a resting potential of -56 mV and cell B -57 mV, and when b values for these cells were calculated from these data and equation (ii) they were 0.08 and 0.07, respectively. Immediately following the addition of KCl, however, the tissue is not in a steady state, so that equation (ii) cannot be used to compare the observed maximal change in potential with that calculated from the maximal $[\text{K}]_e$ of Fig. 5. Instead, the effect may be considered of adopting the equation:

$$V = \frac{RT}{F} \ln \frac{[\text{K}]_e + b[\text{Na}]_e + c[\text{Cl}]_i}{[\text{K}]_i + b[\text{Na}]_i + c[\text{Cl}]_e} \quad (\text{iv})$$

as applied by Hodgkin & Katz (1949) to the squid axon and Hodgkin & Horowicz (1959) to muscle fibres. Here the constant c is a measure of the apparent permeability of Cl^- relative to that of K^+ . It will be seen from this expression that the Cl concentration ratio tends to oppose the depolarization produced by the change in $[\text{K}]_e$.

In making calculations using eqn. (iv) data were obtained as follows. $[\text{K}]_e$ was taken from Fig. 5, having been already calculated using eqn. (i). $[\text{Na}]_e$ was given by the composition of the incubating medium. $[\text{Cl}]_e$ was calculated from eqn. (i), using a diffusion coefficient at 37°C of 2.6×10^{-5} $\text{cm}^2 \text{sec}^{-1}$. The values for $[\text{K}]_i$, $[\text{Na}]_i$ and $[\text{Cl}]_i$ were taken from Table 1, column ii, (a); these are mean values provided by the analysis of a number of tissue slices, and it is assumed that they apply to the particular cells A and B under consideration. It is also assumed that $[\text{K}]_i$ and $[\text{Cl}]_i$ remain constant; this may not be the case, and is discussed more fully later.

If c is taken as 0.5 (close to the value of 0.45 in the squid axon), then for cell A, Fig. 5, the change at maximal $[\text{K}]_e$ would be from -56 mV to -19 mV, close to the observed potential of -22 mV; and for cell B from

-57 mV to -27 mV, the observed potential being -34 mV. The importance of considering Cl permeability and concentration ratio is shown if eqn. (ii) is used to calculate the expected potential change; for cell A the predicted potential is -4 mV, and for cell B -15 mV. Application of eqn. (iv) thus appears justified.

Moreover, in calculating from eqn. (i) the expected K and Cl concentrations, the diffusion path in the tissue has been assumed to be the distance moved by the micro-electrode. The electrode actually travels through cell structures, and the path available for diffusion is greater by a factor which depends on the proportion of tissue volume occupied by cells. Adopting the theoretical treatment of McLennan (1957), cellular volume of 66% (see above) would involve an increase in the diffusion path by 43%. On

TABLE 3. Comparison of observed potential with that calculated from equation (iv) at various times after addition of KCl, for cells A and B of Fig. 5

Time (sec)	Potentials (mV)			
	Cell A		Cell B	
	Observed	Calculated	Observed	Calculated
0	-56	-56	-57	-57
5	-45	-46	-43	-40
10	-37	-35	-36	-34
15	—	—	-34	-32
20	-27	-27	-32	-31
30	-24	-23	-34	-32
40	-23	-22	-35	-33
50	-22	-22	—	—
60	-22	-21	-36	-34
80	-23	-22	—	—

this basis, the potassium concentrations anticipated from eqn. (i) are shown in Fig. 5 as the interrupted lines A (ii) and B (ii). The corresponding potentials from eqn. (iv) with b and c as given above are shown in Table 3. Agreement with the observed values is surprisingly good, particularly in view of the assumptions made regarding constancy of $[K]_i$ and $[Cl]_i$, and the fact that eqn. (i) (which yields values for external ion concentrations) is approximate only.

The tissue is likely, in long-term experiments, to respond to increased external $[K]$ by other changes which would diminish membrane potential. Thus, there is ultimately an adjustment to added $[K]$ by increased intracellular $[K]$; Pappius & Elliott (1956) found such an increase after 60 min incubation. This could, however, have a limited effect only within the 30 sec of the rising phase of Fig. 5. The maximum rate of potassium uptake observed in the tissue was $500 \mu\text{equiv/g/hr}$ (McIlwain, 1963), implying an increase of only $4 \mu\text{equiv/g}$ during 30 sec. The corresponding change in $[K]_i$, from 136 to 143 mM, would have little effect on membrane potential

but is presumably coupled with a greater change in extracellular $[K]$. The joint effect on membrane potential resulting from the two changes could be appreciable but difficult to evaluate as K diffusion would secondarily increase. As far as the constancy of $[Cl]_i$ is concerned, it has been assumed that Cl is in equilibrium at the observed resting membrane potential, thus giving a $[Cl]_i$ of 14 mM (Table 1, column ii, (a)); as soon as the membrane is depolarized to a more positive value than this equilibrium potential, Cl will enter the cell. This may have little effect during the rising phase of the depolarization, but it would be expected to delay the recovery of potential towards the resting value. If, however, chloride is not in equilibrium at the resting membrane potential, and $[Cl]_i$ approaches 33 mM as shown in Table 1, column ii (b), that is the Cl concentration in the non-inulin space, then Cl need not enter the cell until the potential has reached -40 mV. In this case, the 'calculated' potentials of Table 3 will be some 2–3 mV less negative than those given, though still in quite good agreement with the observed values.

The curves of Fig. 5 show also that maximum tissue depolarization occurs some seconds after $[K]_e$ is calculated by eqn. (i) to have reached its maximal value. Two of the factors just discussed could contribute to this delay: the increased length of diffusion path, and potassium uptake by the tissue. Figure 5 shows that the 43% increase in diffusion path alters the time calculated to reach maximal $[K]_e$ from 45 to 60 sec in the case of cell A, or closer to the observed value of 55 sec. With cell B, the 15 sec of Fig. 5 curve (i) became 20 sec; observed value 23 sec. This aspect of diffusion in the extracellular space of the tissue thus appears understandable without the necessity of invoking a large change in the diffusion coefficient of K^+ from that obtaining in free solution. Markedly slower diffusion of K^+ was reported by McLennan (1957) in cerebral tissues which were examined *in vitro* but which were 2 mm in thickness, and thus inadequately oxygenated. A diminution in the diffusion coefficient of K from 2.5×10^{-5} cm² sec⁻¹ to 1.5×10^{-5} cm² sec⁻¹ alters the time calculated to reach maximal $[K]_e$ (cell A) from 45 to 60 sec, an effect similar to that produced by increased diffusion path.

Glutamate

Certain of the factors conditioning diffusion of K^+ in the present tissue condition also the diffusion of glutamate, and indeed the time course of the change in potential after addition of glutamate is similar to that following K^+ , and closely akin to a similarly calculated time course of change in concentration (Fig. 8). If, however, glutamate diffusion travels along a path lengthened by a factor of 1.4 by cell structure (see above) the calculated peak concentration comes 5 sec after that observed.

Accepting the computed concentrations, Fig. 8 indicates little detectable time-lag in the onset of glutamate action, apparently not greater than 1–2 sec. Krnjević & Phillis (1963) concluded that the latency in excitation of the cortex *in situ* by glutamate was also seldom more than 1 sec. Coincidence in the threshold concentrations for action in the two systems was noted above, and the findings thus lead to expectation of a similar mechanism for the two effects. We agree with Krnjević & Phillis's (1963) suggestion of glutamate action at the cell surface rather than intracellularly for the reasons they adduce and also through consideration of metabolic rates. Thus although addition of glutamate to cerebral tissues can lower energy-rich phosphates by some 30% in 30 sec (Woodman & McIlwain, 1961), and so disturb transport mechanisms dependent on energy-rich intermediates, diminution during the 1–2 sec of the present experiments would be no more than 2–3%. If this process is to afford a basis for the action of Fig. 8, siting of the utilization of phosphate close to the point of its utilization in transport would seem necessary. This represents, however, one fashion only in which changed ion permeability may be caused by glutamate (see Krnjević & Phillis, 1963; Eccles, 1964). Dispersion of glutamate by diffusion appears adequate to account for the initial repolarization of the tissue after small glutamate additions, but the metabolic consequences just discussed are likely to contribute to the results of repeated additions of greater quantities. Thus Woodman & McIlwain (1961) found 15–30 mM glutamate to result in fall of phosphocreatine to 0.2–0.5 of its normal value, in 3–10 min, the fall being sustained for at least 40 min. Although glutamate under comparable conditions leads to increased potassium becoming associated with a given initial weight of tissue, the tissue swells and its potassium concentration falls by 15% (Pappius & Elliott, 1956). Possibly these changes lead to the more permanent loss of polarization noted above, to occur as a delayed effect of higher glutamate concentration.

Additions of KCl or of glutamate comparable to those of the present experiments but made to the cerebral cortex *in situ* may not only depolarize locally but also initiate spreading depression (Leão, 1944; Grafstein, 1956; Van Harreveld & Schädé, 1960). This has not been observed in the present system nor do these or larger additions cause *in vitro* the cell discharge induced *in vivo* and associated with the advancing front of the wave of depression. Present observations do, however, lend support to mechanisms of spreading depression which depend on mediation by liberated K^+ or glutamate, its accumulation extracellularly, and diffusion to excite adjacent regions (see Burns, 1958; Ochs, 1962; Brinley, 1963) for the time course of a wave of spreading depression is of the order of magnitude of the changes of Fig. 5 or 8.

SUMMARY

1. Apparatus is described for maintaining slices of mammalian cerebral cortex under good metabolic conditions while continuous recordings were made of membrane potential, with micropipette electrodes.

2. The average stable resting potential observed was 59 ± 10 mV (S.D.). Potentials recorded in media 2.6 mM in calcium were more stable than those in media containing 0.75 mM calcium.

3. Measured amounts of KCl in about 1 μ l. of fluid were applied to the tissue surface. They diminished the resting potential, which subsequently recovered. The course of this change was compared with changes in potassium concentration near the cell calculated to result by diffusion.

4. Addition to the tissue surface of sodium glutamate in small volumes of fluid also resulted in depolarization of cell elements, followed by recovery; a similar comparison was made of glutamate concentration and potential change.

5. The sodium, potassium and chloride of the tissues were determined before and after incubation; in some cases incubation media contained inulin and this also was determined. The volume and ion content of the intracellular compartment of the tissue was computed and the gradients in sodium, potassium and chloride compared with the observed membrane potentials.

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