Glutamate, Calcium Ion-Chelating Agents and the Sodium and Potassium Ion Contents of Tissues from the Brain

By I. PULL, H. McILWAIN AND R. L. RAMSAY

Department of Biochemistry, Institute of Psychiatry (British Postgraduate Medical Federation, University of London), De Crespigny Park, London S.E.5, U.K.

(Received 27 June 1969)

1. Salts of L-glutamate added to cerebral tissues maintained in glucose-saline-bicarbonate solutions cause the Na^+ content of the tissues to increase rapidly and K^+ to be lost. Entry of $^{22}\text{Na}^+$ also is accelerated by L-glutamate and this acceleration is inhibited by low concentrations of tetrodotoxin. 2. Tissue Na^+ content and its rate of increase after the addition of L-glutamate are affected by the Ca^{2+} of incubation media. 3. Very rapid and extensive entry of Na^+ to the tissue is caused by EDTA, and a moderate entry by citrate and ATP. Calculations of the concentration of free Ca^{2+} in media after these additions indicate that Na^+ entry is sometimes associated with low Ca^{2+} concentration, but that other substances, especially L-glutamate, act without greatly diminishing Ca^{2+} concentration. 4. Experiments with 2,4-dinitrophenol and valinomycin are also reported and aspects of the Na^+ entry formulated and discussed.

The ordinarily high K^+ and low Na^+ contents of cerebral tissues *in vivo* are maintained *in vitro*, but undergo rapid change when the tissues are electrically excited (Cummins & McIlwain, 1961; Bachelard, Campbell & McIlwain, 1962). Addition of glutamate causes comparably rapid Na^+ entry and loss of K^+ , and these movements, initiated electrically or by glutamate, are opposed by tetrodotoxin (McIlwain, Harvey & Rodriguez, 1969). These results suggest that tetrodotoxin and glutamate both act at membrane sites that are primarily concerned with the entry of Na^+ during the nerve impulse. It has already been proposed, in other neural systems, that Ca^{2+} interacts with such sites (see Frankenhaeuser & Hodgkin, 1957; Koketsu, 1965; Ames, Tsukada & Nesbett, 1967). Also, agents that could chelate with Ca^{2+} show some similarities to glutamate in their actions on certain cerebral cells (Galindo, Krnjevic & Schwartz, 1967).

We have therefore compared glutamate with the chelating agents for action on cerebral cations. Movements of Na^+ and K^+ , induced in these and in other ways, have also been examined for their susceptibility to tetrodotoxin.

EXPERIMENTAL

Tissue preparation and incubation. Guinea pigs were struck on the neck, exsanguinated, their cerebral hemispheres were removed and two slices (0.35 mm) prepared with a bow-cutter from each hemisphere as described by McIlwain & Rodnight (1962) and Harvey & McIlwain (1968). Immediately after being cut, each slice was

weighed and placed in incubation medium at 37.5°C and agitated by a stream of $\text{O}_2 + \text{CO}_2$ (95:5), in 30 ml beakers provided with well-fitting plastic lids with small vents for gas egress. Standard incubation media contained: NaCl, 124 mM; KCl, 5 mM; KH_2PO_4 , 1.24 mM; MgSO_4 , 1.3 mM; NaHCO_3 , 26 mM; CaCl_2 , 0.75 mM; glucose, 10 mM; inulin, 1%. Modifications to the media before and at specified times during incubation, and transfer of tissues to fresh batches of media, are noted in individual experiments. Incubation was terminated by lifting the tissues successively from their beakers with a bent wire, and then immediately removing excess of incubation fluid by contact with a glass surface before reweighing the tissues and placing them in extracting fluids.

Analytical methods. The normal extracting fluid was 6% (w/v) trichloroacetic acid (4 ml) in a test-tube homogenizer, in which the tissue was ground and the mixture kept for 30 min at room temperature. Supernatants were taken for determination of Na, ^{22}Na and K as described by Harvey & McIlwain (1968), and of inulin as described below.

Autoanalytical method for inulin. To determine inulin in the tissue extracts and incubation media, the methods of Dawborn (1965) and Wright & Gann (1966) were examined but were found less suitable than the following method, which adapts the procedure of Varon & McIlwain (1961) to a Technicon Instruments Corp. AutoAnalyzer. The flow diagram for the determination is shown in Fig. 1; the acid reagent was 0.46 mM- FeCl_3 in conc. HCl and the resorcinol reagent was 0.1% (w/v) resorcinol in 50% (v/v) acetic acid; standard inulin solutions contained 5–30 μg of inulin/ml. Table 1 shows satisfactory recovery of standard amounts of inulin in tissue extracts and samples of media, judged between and within replicate analyses.

Expression of results. Tissue analysis was first expressed as quantities per unit wt. of tissue; from analysis of the

Fig. 1. Flow diagram for the determination of inulin with the Technicon AutoAnalyzer. Samples (40/h) are from a Sampler 11 with a sample/wash ratio 1:1. Flow rates are shown in ml/min. A, The sample; B, the acid reagent; C, air; D the resorcinol reagent. DMC represents a double mixing coil and HB the heating bath, only one coil of which is used. Transmission tubing is glass or Acidflex.

$$K_{ML}/K'_{ML} = [L_{total}]/[L] = 1 + [H^+]/K_4 + [H^+]^2/K_4 K_3 + [H^+]^3/K_4 K_3 K_2 + [H^+]^4/K_4 K_3 K_2 K_1$$

Table 1. *Inulin recovery by the autoanalytical method*

The concentrations of inulin already present in tissue extracts and samples of media were increased by adding known amounts of inulin, and the resulting concentrations were determined. Four experiments were carried out over a period of several months. Results are expressed as mean percentage recovery of the added inulin $\pm$ S.D. of five replicates.

Expt. no.	Recovery of added inulin (%)		
	Inulin added to tissue extracts		Inulin added to media samples
	(5 μ g/ml)	(10 μ g/ml)	(5 μ g/ml)
1	99.2 $\pm$ 2.0	98.3 $\pm$ 0.2	99.4 $\pm$ 0.4
2	102.5 $\pm$ 0.6	104.4 $\pm$ 1.2	99.7 $\pm$ 0.0
3	98.6 $\pm$ 0.3	99.4 $\pm$ 0.5	100.4 $\pm$ 0.5
4	97.8 $\pm$ 0.3	97.7 $\pm$ 0.4	99.1 $\pm$ 0.5
Mean	99.5 $\pm$ 1.8	100.0 $\pm$ 2.5	99.7 $\pm$ 0.5

media, the tissue inulin content was expressed as an inulin space (μ l/g) and the Na, 22 Na and K in the residual non-inulin space were calculated by the methods and assumptions of Harvey & McIlwain (1968).

Calculation of Ca^{2+} and Mg^{2+} concentrations. Constants for the calculations of Ca^{2+} and Mg^{2+} concentrations in the presence of the ligands used are given in Table 2; only complexes of the type shown are considered. The association constants are given by:

$$K_{ML} = [ML]/[M][L]; K_{MHL} = [MHL]/[M][HL] \quad (1)$$

where L represents the ligand and M is either Mg^{2+} or Ca^{2+} ; the particular equation used depends on the type of complex formed.

With EDTA and EGTA,* conditional association constants at pH 7.6 are defined as:

$$K'_{ML} = [ML]/[M][L_{total}] \quad (2)$$

where L_{total} is the total ligand not complexed by the metal and is the sum of L, HL, H_2L , H_3L and H_4L . The relationship between the association constants and the ligand species is given by:

where $[H^+]$ is the H^+ concentration and K_1, K_2, K_3 and K_4 are acid dissociation constants for the ligand. No corrections have been applied to the other ligands since their metal complexing species preponderate at pH 7.6 and eqn. (1) is therefore used.

Since Ca^{2+} and Mg^{2+} are both present, free-ion concentrations are calculated to a first approximation by applying eqn. (1) or eqn. (2) in an iterative process (Portzehl, Caldwell & Ruegg, 1964).

Materials. Tetrodotoxin was obtained from the Sankyo Co., Tokyo, Japan, as a mixture of pure crystalline compound with sodium citrate buffer. Quantities (about 0.1 mg) dissolved in a few millilitres of water were weighed by electrobalance and dispensed by micropipettes or kept at $-20^\circ C$. 6-N-2'-O-Dibutyladenosine 3':5'-cyclic phosphate was from Calbiochem Ltd., London W.1, U.K., as a hydrated sodium salt. Valinomycin was from Dr J. C. MacDonald, Saskatoon, Sask., Canada (MacDonald, 1960), and was dispensed by solution in ethanol, dilution to 4%.

* Abbreviation: EGTA, ethanedioxybis(ethylamine)-tetra-acetate.

Table 2. *Association constants used for calculations of Ca^{2+} concentrations*

Values were selected from Sillen & Martell (1964) for ionic strength 0.1–0.2 and $20^\circ C$ except for citric acid, phosphoric acid and glutamic acid, which are values at $25^\circ C$. L and M represent ligand and metal ion respectively; for details see the text.

Ligand	Type	Acid dissociation constant				Complex	Association constant		Conditional association constant, pH 7.6	
		pK_1	pK_2	pK_3	pK_4		$\log K_{Ca^{2+}}$	$\log K_{Mg^{2+}}$	$\log K'_{Ca^{2+}}$	$\log K'_{Mg^{2+}}$
EGTA	H_4L	2.0	2.68	8.85	9.46	ML^{2-}	11.0	5.21	7.87	2.08
EDTA	H_4L	1.99	2.67	6.16	10.26	ML^{2-}	10.7	8.69	8.02	6.01
Citric acid	H_4L	2.96	4.39	5.67	10.82	MHL^-	4.84	3.29	—	—
ATP	H_4L	—	—	4.05	6.5	ML^{2-}	3.6	4.0	—	—
Glutamic acid	H_3L	2.30	4.28	9.67	—	MHL	1.43	1.9	—	—
Phosphoric acid	H_3L	2.4	6.6	11.1	—	MHL	1.70	1.88	—	—

Table 3. *Neocortical Na⁺ and K⁺ contents after incubation in modified media*

Samples from the outer 350 μm of guinea-pig neocortex were incubated for 40 min in the standard bicarbonate-glucose-saline medium containing 0.75 mM-Ca²⁺ and inulin. Tetrodotoxin, when indicated, was present throughout the experiment. Some tissues of group (a) were transferred to a new batch of the same medium after the initial 40 min incubation. The tissues in groups (b) and (c) were rinsed in Ca²⁺-free media and then incubated further in the Ca²⁺-free media. In groups (d), (e) and (f) incubation continued in the original medium but the additions specified were made at 40 min. Values in group (a) are the means $\pm$ s.d. of six results, in groups (c) and (f) at 8 min are of four results and the others are the means of duplicate results.

Group of expt.	Incubation medium			Tissue non-inulin space	
	Change in medium (mM)	Tissue in changed medium (min)	Tetrodotoxin present (μM)	Na ⁺	K ⁺
				($\mu\text{equiv./g}$)	($\mu\text{equiv./g}$)
(a)	None	0 or 60	0	15 $\pm$ 3.2	71 $\pm$ 2.3
	None	0 or 60	3.1	14 $\pm$ 5.1	74 $\pm$ 2.1
(b)	No Ca ²⁺	10	0	19	69
	No Ca ²⁺	10	3.1	20	68
(c)	No Ca ²⁺	30 or 60	0	30 $\pm$ 3.1	65 $\pm$ 2.5
	No Ca ²⁺	30 or 60	3.1	22 $\pm$ 4	70 $\pm$ 2.8
(d)	EDTA (5)	5	0	100	28
	EGTA (5)	5	0	12	70
	EGTA (11)	5	0	59	59
	ATP (4)	15	0	20	66
	ATP (10)	15	0	35	52
(e)	EDTA (5)	1	0	37	56
	EDTA (5)	1	3.1	30	59
	EDTA (5)	2	0	61	37
	EDTA (5)	2	3.1	50	52
(f)	Dinitrophenol (0.3)	8	0	37 $\pm$ 6	54 $\pm$ 2.5
	Dinitrophenol (0.3)	8	0.06	31 $\pm$ 5.5	52 $\pm$ 3
	Dinitrophenol (0.3)	15	0	56	46
	Dinitrophenol (0.3)	15	0.06	42	48

(v/v) ethanol and immediate addition to the reaction mixture; the final ethanol concentration was 0.2% (v/v). Ethanol to this concentration was also added to control media. ²²NaCl was obtained as an iso-osmotic solution from The Radiochemical Centre, Amersham, Bucks., U.K. Other substances were analytical reagents or the highest grade of laboratory reagents.

RESULTS

Ca²⁺-free media and chelating agents. When normal values are re-established by incubation for about 30 min, the Na⁺ and K⁺ content of cerebral tissues can remain constant during subsequent periods for 1 h or more (Table 3). Small changes only were brought about by transferring the tissue samples from normal media, 0.75 mM in calcium salts, to Ca²⁺-free media. At $\frac{1}{2}$ –1 h after such transfer the tissues had gained about 12 $\mu\text{equiv.}$ of Na⁺ (non-inulin)/g and lost 5 $\mu\text{equiv.}$ of K⁺/g.

Chelating agents, by contrast, brought about much more rapid and extensive changes (Table 3). Within 5 min of the addition of 5 mM-EDTA the tissue Na⁺ content was increased by 85 $\mu\text{equiv./g.}$

Tissue K⁺ content decreased by 53 $\mu\text{equiv./g}$ in this time. EGTA was needed in greater amounts to cause rapid changes. The initial speed of net entry of Na⁺ caused by EDTA was at least 1100 $\mu\text{equiv./h}$ per g (Fig. 2). Under similar experimental conditions glutamate could initiate an entry of 450 $\mu\text{equiv.}$ of Na⁺/h per g. Both citrate and ATP increased the tissue Na⁺ content and diminished that of K⁺, but they were less effective than EDTA (Table 4).

Tetrodotoxin and the cation changes induced by alterations in Ca²⁺ concentration. The Na⁺ and K⁺ contents of tissues incubated *in vitro* under the normal conditions, which afford maximal intracellular K⁺ and minimal Na⁺ contents, were not significantly affected by tetrodotoxin (Table 3 and Fig. 3). Conditions for maximal K⁺ and minimal Na⁺ contents have been carefully examined (Bachelard *et al.* 1962; Lolley & McIlwain, 1964; Keeseey, Wallgren & McIlwain, 1965) and the optimum conditions include a narrowly defined Ca²⁺ concentration of 0.75 mM in incubation media. When this concentration is diminished or increased

Fig. 2. Na^+ content (a) and K^+ content (b) of the non-inulin space of cortical tissues. To tissues preincubated as described in Table 3, solutions of reagents were added at 40 min (zero time in the figure) to give in the incubation media the millimolar concentrations given in parentheses (Glu, glutamate; Cit, citrate). At 2 or 5 min later the tissues were removed with a bent wire, reweighed and transferred to trichloroacetic acid for analysis. Experimental points are means of two values, except that at zero time six values are averaged and the s.d. of the means quoted.

the tissue Na^+ content increases. The values after 60 min in Table 3 and the zero-time value after 30 min preincubation in Fig. 3 show that this Na^+ entry is susceptible to tetrodotoxin. Values with 0.75 mM-Ca^{2+} in Fig. 3 agree with those of McIlwain *et al.* (1969).

Tissues incubated in media with 2.8 mM-Ca^{2+} underwent a smaller net change in Na^+ and K^+ contents on the addition of glutamate (Fig. 3) than did those in 0.75 mM-Ca^{2+} ; $60 \text{ nM-tetrodotoxin}$ further limited the cation movements. To this extent, therefore, tetrodotoxin and calcium salts are acting synergistically.

$3.1 \mu\text{M-Tetrodotoxin}$ partially retarded the changes in Na^+ and K^+ contents caused by the addition of EDTA to neocortical tissues in media containing 0.75 mM-Ca^{2+} . This was most evident at 2 min after the addition of EDTA (Table 3).

Interacting concentrations of glutamate and tetrodotoxin. McIlwain *et al.* (1969) reported that tetrodotoxin opposes the action of glutamate in altering the tissue Na^+ and K^+ contents, at one concentration; the present work reports the effects of the two reagents over a 25-fold range in concentration (Fig. 4), and also examines the entry of $^{22}\text{Na}^+$ into the tissue samples. After preincubation some of the tissues received tetrodotoxin and subsequently glutamate with or without $^{22}\text{NaCl}$, and were removed for

Fig. 3. Effect of glutamate on neocortical non-inulin-space Na^+ (a) and K^+ (b). The procedure was that employed in the experiments of Fig. 2, except that half the media (●) contained $60 \text{ nM-tetrodotoxin}$ throughout the incubation. ---, 0.75 mM-Ca^{2+} ; —, 2.8 mM-Ca^{2+} . Lines extend from points giving the s.d. of four or six values; other points are means of duplicate results.

analysis 2 min after the final addition. This time-interval was chosen on the basis of the experiments of Fig. 3. Tetrodotoxin at 330 nM completely prevented the additional entry of ^{22}Na brought about by the glutamate, whereas the increase in Na^+ content was only partially diminished and K^+ was little affected (Fig. 4).

Other observations. Neocortical Na^+ content was increased and that of K^+ decreased by 2,4-dinitrophenol (Table 3), presumably through its known effects on energy metabolism. The change with dinitrophenol was slower than with glutamate or with EDTA, and 8 min after the addition of dinitrophenol was unaffected by tetrodotoxin; some antagonism occurred after 15 min.

Electrical stimulation of neocortical tissues causes a several-fold increase in their content of adenosine 3':5'-cyclic phosphate (Kakiuchi, Rall & McIlwain, 1969); this compound might mediate in excitation and its dibutyl derivative enter cells (Bdolah & Schramm, 1965; Chase & Aurbach, 1967). A solution of the monosodium salt of 6-N-2'-O-dibutyladenosine 3':5'-cyclic phosphate was added to the media in which neocortical tissues were incubated under the conditions given in Fig. 2. At 3 and 30 min later, tissues were sampled for Na^+ , K^+ and inulin contents, but the Na^+ and K^+ of the non-inulin space were unaffected by the added compound. Valinomycin, though affecting cation permeability in other systems (Hofer & Pressman, 1966; Ogata & Rasmussen, 1966), had little or no action on the Na^+ and K^+ contents of neocortical

Fig. 4. Effects of tetrodotoxin and glutamate on neocortical non-inulin-space K^+ (a), Na^+ (b) and $^{22}\text{Na}^+$ (c). To tissues preincubated as described in Table 3 tetrodotoxin or saline was added at 30 min, and sodium glutamate at 45 min to give the millimolar concentrations given in parentheses. At 47 min the tissues were removed for analysis.

tissues when added to them at 0.1 or 1 $\mu\text{g}/\text{ml}$, with and without 0.62 μM -tetrodotoxin, under the conditions of Table 3.

DISCUSSION

Present results on the mechanism of Na^+ entry in neural excitatory processes are considered below in relation to studies on membrane polarization and cell-firing in spinal and cerebral systems, when several of the reagents used here were examined.

(1) The Na^+ entry after the addition of 5mM-EDTA exceeds in speed and extent the maximal entry induced by electrical stimulation of neocortical tissue. With EDTA the rate reached 1350 $\mu\text{equiv.}$ of Na^+/h per g of tissue, and this value indicates that the entry caused electrically, of some 500–600 $\mu\text{equiv.}/\text{h}$ per g (Keesey *et al.* 1965), though rapid, is nevertheless still controlled.

(2) The finding that the maximal Na^+ entry after the addition of glutamate is also about 600 $\mu\text{equiv.}/\text{h}$ per g (Harvey & McIlwain, 1968) is thus additionally significant since it supports the possibility that glutamate is involved in normal neural excitation.

(3) Citrate (10 or 30mM) and ATP (4 or 10mM) each had greater Ca^{2+} -chelating ability than glutamate, but nevertheless, were less potent than glutamate in allowing Na^+ entry. This is expressed quantitatively in Table 4, which shows that citrate and ATP were used in concentrations that diminished the Ca^{2+} concentration to which the tissues were exposed to values of 0.1 or 0.01 of those caused by added glutamate. Nevertheless, glutamate caused by far the greatest Na^+ entry, and therefore, as an inducer of Na^+ entry, probably has specific properties beyond those of a Ca^{2+} -chelating agent. Conclusions that are in part similar have been reached in experiments to compare the action of glutamate and of chelating agents on cell-firing by spinal neurons (Curtis, Perrin & Watkins, 1960) and by neurons of the cuneate nucleus (Galindo *et al.* 1967). Cognate properties in amino acids may be much more widespread: rates of Na^+ entry into ascites cells induced by glycine (Eddy, 1968) are similar to those described above that are caused by glutamate in neocortical tissues.

(4) Glutamate apart, the results obtained with the chelating agents EDTA and EGTA (Table 4) indicate that, for major increase in Na^+ entry both Ca^{2+} and Mg^{2+} must be largely complexed. Rapid entry of Na^+ occurred with EDTA when the concentration of EDTA was above 2mM, equivalent to the sum of the Ca^{2+} (0.75mM) and Mg^{2+} (1.3mM) present in the same solution. Greater concentrations of EGTA were required; this compound does not, however, diminish $[\text{Mg}^{2+}]$ as effectively as does EDTA (Table 4).

(5) Observations with tetrodotoxin also differentiate processes of Na^+ entry initiated by various agents (see Kao, 1966; Moore & Narahashi, 1967). In low concentration (50–100nM) the toxin inhibits almost completely the electrically induced Na^+ entry to neocortical tissues (McIlwain *et al.* 1969), but inhibits only partly the Na^+ entry induced by EDTA, glutamate or dinitrophenol. The specific mechanism of Na^+ entry on electrical stimulation may thus be involved only partly in the Na^+ entry induced by these other means. The $^{22}\text{Na}^+$ entry after the addition of glutamate in the experiments of Fig. 4 was, however, strongly inhibited by tetrodotoxin; in this respect the entry is like that induced electrically.

(6) Synergistic relationships between tetrodotoxin and Ca^{2+} agree with previous work suggesting that Ca^{2+} acts at points of Na^+ entry. Detailed evidence (Tobias, 1964) involves phosphatidylserine in the

Table 4. *Observed tissue Na⁺ contents and calculated Ca²⁺ and Mg²⁺ concentrations in the medium after specified additions*

Free Ca²⁺ and Mg²⁺ are calculated from the data of Table 2 as described in the Experimental section. Tissue Na⁺ contents are quoted as means $\pm$ S.D. of four values, or means of two values.

Addition (mm)	Free [Ca ²⁺] (M)	Free [Mg ²⁺] (M)	Na ⁺ of tissue 5 min after addition (μ equiv./g)
None	7.5×10^{-4} (7.1×10^{-4})*	1.3×10^{-3} (1.2×10^{-3})*	21 $\pm$ 3
Glutamate (5)	6.7×10^{-4} (6.3×10^{-4})*	9.5×10^{-4} (8.6×10^{-4})*	66 $\pm$ 4.5
ATP (4)	7.8×10^{-5}	5.9×10^{-5}	20†
ATP (10)	2.2×10^{-5}	1.6×10^{-5}	35†
Citrate (10)	2.5×10^{-6}	7.8×10^{-5}	27
Citrate (30)	1.0×10^{-6}	2.3×10^{-5}	32
EDTA (1)	2.4×10^{-6}	1.0×10^{-3}	19 $\pm$ 3
EDTA (2)	3.9×10^{-7}	6.7×10^{-5}	18
EDTA (3.5)	4.6×10^{-9}	8.7×10^{-7}	81
EDTA (5)	2.4×10^{-9}	4.3×10^{-7}	96 $\pm$ 4
EDTA (7)	1.7×10^{-9}	2.4×10^{-7}	83
EGTA (1)	3.9×10^{-9}	1.2×10^{-3}	26
EGTA (5)	2.7×10^{-9}	8.3×10^{-4}	23
EGTA (11)	1.1×10^{-9}	5.3×10^{-4}	59

* Allowing for formation of CaHPO₄ and MgHPO₄.

† 15 min after addition.

actions of Ca²⁺ in conditioning the ion movements of neural excitation, and phosphatidylserine-protein complexes have been invoked as a site of action of glutamate as excitant (Watkins, 1965) and of tetrodotoxin as inhibitor (McIlwain *et al.* 1969).

(7) Though actions of tetrodotoxin and glutamate on Na⁺ entry are opposed, the effects of a range of concentrations of each do not suggest that the compounds compete for a common site. This is consistent with the previous proposal (McIlwain *et al.* 1969) that they interact with tissue components. Two suggestions may be offered for the ability of added glutamate to simulate depolarization in allowing Na⁺ entry. (i) The depolarization may allow redistribution of the pre-existing glutamyl groups of peptides lining a transmembrane pore, as proposed by McIlwain (1963). (ii) The membrane depolarization may allow the tissue's native glutamate to penetrate the membrane pore from its inner mouth. Proposal (ii) has points in common with a suggestion (Hodgkin, 1965) that similarities in neural firing induced by depolarization and by removal of Ca²⁺ could be due to the membrane potential holding Ca²⁺ at the neural membrane. In the present system, as in those discussed by Hodgkin (1965), an alternative to the specific role proposed for Ca²⁺ is that it might act by attachment as a counterion to a major part of the acidic groups of neuronal outer cell membranes, and in this way limit the access of Na⁺.

We thank the Research Fund of the Bethlem Royal Hospital and the Maudsley Hospital for support for this

work. R.L.R. held a Postdoctoral Research Fellowship from the National Council to Combat Blindness Inc., New York, N.Y., U.S.A. We thank Dr J. C. MacDonald for a gift of valinomycin.

REFERENCES

- Ames, A., Tsukada, Y. & Nesbett, F. B. (1967). *J. Neurochem.* **14**, 145.
 Bachelard, H. S., Campbell, W. J. & McIlwain, H. (1962). *Biochem. J.* **84**, 225.
 Bdoah, A. & Schramm, M. (1965). *Biochem. biophys. Res. Commun.* **18**, 452.
 Chase, L. R. & Aurbach, G. D. (1967). *Proc. natn. Acad. Sci. U.S.A.* **58**, 518.
 Cummins, J. T. & McIlwain, H. (1961). *Biochem. J.* **79**, 330.
 Curtis, D. R., Perrin, D. D. & Watkins, J. C. (1960). *J. Neurochem.* **6**, 1.
 Dawborn, J. K. (1965). *Clinica chim. Acta*, **12**, 63.
 Eddy, A. A. (1968). *Biochem. J.* **108**, 195.
 Frankenhaeuser, B. & Hodgkin, A. L. (1957). *J. Physiol., Lond.*, **137**, 218.
 Galindo, A., Krnjevic, K. & Schwartz, S. (1967). *J. Physiol., Lond.*, **192**, 359.
 Harvey, J. A. & McIlwain, H. (1968). *Biochem. J.* **108**, 269.
 Hodgkin, A. L. (1965). *The Conduction of the Nervous Impulse*, p. 68. Liverpool University Press.
 Hofer, M. & Pressman, B. C. (1966). *Biochemistry, Easton*, **5**, 3919.
 Kakiuchi, S., Rall, T. W. & McIlwain, H. (1969). *J. Neurochem.* **16**, 485.
 Kao, C. Y. (1966). *Pharmac. Rev.* **18**, 997.
 Keesey, J. C., Wallgren, H. & McIlwain, H. (1965). *Biochem. J.* **95**, 289.

- Koketsu, K. (1965). In *Studies in Physiology*, p. 125. Ed. by Curtis, D. R. & McIntyre, A. K. Berlin: Springer-Verlag.
- Lolley, R. N. & McIlwain, H. (1964). *Biochem. J.* **93**, 128.
- MacDonald, J. C. (1960). *Can. J. Microbiol.* **6**, 27.
- McIlwain, H. (1963). *Chemical Exploration of the Brain: A Study of Cerebral Excitability and Ion Movement*. Amsterdam: Elsevier Publishing Co.
- McIlwain, H., Harvey, J. A. & Rodriguez, G. (1969). *J. Neurochem.* **16**, 363.
- McIlwain, H. & Rodnight, R. (1962). *Practical Neurochemistry*. London: J. and A. Churchill Ltd.
- Moore, J. W. & Narahashi, J. (1967). *Fedn Proc. Fedn Am. Soc. exp. Biol.* **26**, 1655.
- Ogata, E. & Rasmussen, H. (1966). *Biochemistry*, Easton, **5**, 57.
- Portzehl, H., Caldwell, P. C. & Ruegg, J. C. (1964). *Biochim. biophys. Acta*, **79**, 581.
- Sillen, L. G., & Martell, A. E. (1964). *Spec. Publ. chem. Soc.* no. 17: *Stability Constants of Metal Ion Complexes*.
- Tobias, J. M. (1964). *Nature, Lond.*, **203**, 13.
- Varon, S. & McIlwain, H. (1961). *J. Neurochem.* **8**, 262.
- Watkins, J. C. (1965). *J. theor. Biol.* **9**, 37.
- Wright, H. K. & Gann, D. S. (1966). *J. Lab. clin. Med.* **67**, 689.