The Sodium, Potassium and Chloride of Cerebral Tissues: Maintenance, Change on Stimulation and Subsequent Recovery

By J. C. KEESEY, H. WALLGREN AND H. McILWAIN

Department of Biochemistry, Institute of Psychiatry (British Postgraduate Medical Federation, University of London), Maudsley Hospital, London, S.E. 5

(Received 22 June 1964)

1. Cerebral tissues were prepared for incubation by cutting them from the brain rapidly and *in situ*, and the calcium concentration in the incubating medium was altered from the customary 2.8 mM to 0.75 mM. This provided incubated cerebral cortex with fluid and ion content more closely resembling that of the brain *in vivo* than hitherto obtained. 2. From a systematic difference in size between inulin spaces of slices with one and those with two cut surfaces, it was estimated that cutting directly affected a layer 0.02 mm. thick. On the basis of the volume of this layer, it was calculated that the portion of the tissue not affected by cutting had an inulin space of 258 μ l./g. initial wt., and during the process of preparation and incubation had gained 30 μ equiv. of sodium and 17 μ equiv. of chloride/g. and had lost 14 μ equiv. of potassium/g. 3. Several aspects of the ion content of the incubated tissue were compatible with the observed membrane potential of -60 mV between cellular and extracellular phases. 4. In response to electrical stimulation, sodium of the non-inulin space increased from 28 to 57 μ equiv./g., potassium decreased from 68 to 48 μ equiv./g. and chloride increased from 16 to 22 μ equiv./g. in the non-inulin space. These changes were complete in about 6 min., and thereafter the concentrations remained steady during continued stimulation. Initial rates of change were 460 μ equiv./g./hr. for sodium and 480 μ equiv./g./hr. for potassium. 5. After stimulation was stopped the ionic composition of the tissue returned completely to its pre-stimulation state within 10 min. Initial rates for extrusion of sodium and gain of potassium were 160 and 230 μ equiv./g./hr. respectively.

Isolated cerebral tissues maintained *in vitro* have been shown by electrical and by many metabolic measurements to respond to electrical pulses, as do other neural systems (McIlwain, 1963). Electrical stimulation is regarded as causing, by purely electrical means, sufficient depolarization of the membranes of excitable cells of the tissue for them to alter in permeability as do other neural membranes (see Eccles, 1957; Hodgkin, 1958), and permit Na^+ ions of external fluids to enter the cells, followed within a fraction of a millisecond by outflow from the cells of K^+ ions. By the use of intracellular micro-pipettes Hillman, Campbell & McIlwain (1963) demonstrated depolarization of individual cells almost to zero potential on application of electrical pulses to isolated slices of guinea-pig cerebral cortex, and also showed restoration of normal resting potentials after termination of pulses.

The corresponding changes in tissue content of sodium and potassium in response to electrical stimulation have also been reported (Varon &

McIlwain, 1961; Bachelard, Campbell & McIlwain, 1962; McIlwain & Joanny, 1963; Joanny & Hillman, 1963), and Cummins & McIlwain (1961), using ^{42}K , demonstrated an increased turnover of potassium on electrical stimulation. However, because cerebral-cortex slices as ordinarily handled during experiments *in vitro* lose potassium and gain sodium, chloride and fluid, only a relatively small proportion of the tissue sodium has so far been involved in the response to stimulation. Further, restoration of pre-stimulation concentrations of ions after termination of pulses has been only partial.

Therefore, before proceeding to investigate sodium movements in further detail, it seemed desirable first to obtain incubated cerebral-cortex tissue more closely resembling fresh tissue in ion composition and regulatory capacity. The present paper describes improved methods of preparation and incubation of isolated cerebral-cortex slices, based on the findings of Bachelard *et al.* (1962), Lolley (1963) and Lolley & McIlwain (1964), and

Bioch. 1965, 95

also describes the time-courses of net changes in ion content of such slices in response to the onset and termination of electrical stimulation. Keese & Wallgren (1965) describe measurements made on the movements of radioactive sodium into and out of these slices under similar conditions.

EXPERIMENTAL

Preparation of tissue

A guinea pig was stunned by a blow on the back of the neck and exsanguinated by cutting across the carotid arteries. The bone surrounding the brain was removed as described by Hillman *et al.* (1963) and two slices, each about 0.35 mm. thick, were obtained from each hemisphere by using a completely dry blade and guide (McIlwain & Rodnight, 1962). Each slice was removed from the blade with a bent-wire rider and weighed to the nearest milligram on a torsion balance. The slice was then released from the rider into an evaporating dish containing 50 ml. of glucose-bicarbonate medium maintained at 37° in a water bath. During this procedure a gas mixture of O₂+CO₂ (95:5) was bubbled continuously through this medium. The medium in the open dish tended to lose CO₂ to the atmosphere, and became slightly more alkaline during the procedure. Therefore for this purpose medium was originally made up with less NaHCO₃ (15 mM) to be somewhat more acid (pH 7.4) than the customary glucose-bicarbonate medium (pH 7.65). During the time the slices were in contact with this medium its pH remained approx. 7.65. Four slices weighing 35–65 mg. were obtained in this manner from a single brain within 4–5 min. of stunning the animal.

Incubation. Each slice in turn was taken up from the medium in the evaporating dish by a rapid-transfer holder (McIlwain & Rodnight, 1962) and placed in a 30 ml. beaker containing 5 ml. of glucose-bicarbonate medium at 37° that was, unless otherwise stated, of the following composition: NaCl (124 mM), NaHCO₃ (26 mM), KCl (5 mM), KH₂PO₄ (1.24 mM), CaCl₂ (0.75 mM), MgSO₄ (1.3 mM), glucose (10 mM) and inulin (1%). A gas mixture of O₂+CO₂ (95:5) was bubbled through this medium for 10 min. before the beginning of the experiment and also throughout incubation. All slices were incubated at least 25 min. before being subjected to any further procedures. Electrical stimulation when applied was in the form of 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).

Removal for analysis. The incubated slices were released from their holders into cold rinses of 0.3 M-tris, -glycylglycine or -glucose. Periods even as short as 3 sec. were found sufficient to rinse off adhering medium and also to remove at different rates the ions and inulin from the tissue extracellular space. Similar results were obtained after draining incubated slices by touching their flat surfaces to a clean glass surface. Therefore the method adopted for the present experiments, unless otherwise stated, was as follows: at the end of an experiment each slice was released from its holder into the medium in which it had just been incubated. The slice was then picked out of this medium with a bent-wire rider within 3–6 sec. of release and drained gently up the side of the beaker to remove a drop of adhering fluid before the slice was reweighed.

Analytical methods

After the reweighing, each slice 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. The supernatant fluids were poured into 25 ml. volumetric flasks. The residues were resuspended in 5 ml. of glass-distilled water, centrifuged again and supernatants added to their respective flasks, which were then diluted to 25 ml. with glass-distilled water and shaken before samples were removed for determination of sodium, potassium, chloride and inulin. Total protein was determined in each tissue residue by the biuret method (Gornall, Bardawill & David, 1949) as a check on the initial weight of the slice.

The media in which the slices had been incubated were pooled as soon as the slices had been removed, and from this pooled medium two 50 µl. samples were each added to 4 ml. of 6% trichloroacetic acid. Blanks containing 4 ml. of 6% trichloroacetic acid only and three standards each containing 4 ml. of 6% trichloroacetic acid and known amounts of sodium, potassium, chloride and inulin were treated in a manner identical with that used for the tissue samples, including the use of homogenizers. Internal standards, consisting of standard salt solutions added to tissue extracts, were also analysed.

Glassware. All homogenizers, flasks and glassware used for analysis of sodium, potassium and chloride were soaked overnight in 2 N-HNO₃, rinsed four times with distilled water and three times with glass-distilled water, dried in an oven at 105° and then used immediately. The same HNO₃ was used for two experiments before being discarded.

Sodium and potassium. Duplicate samples (2 ml.) from each flask were taken and added to 4 ml. of glass-distilled water, and the sodium and potassium concentrations determined with a flame photometer (model A; Evans Electro-selenium Ltd., Halstead, Essex) with the appropriate filters. The standards contained Na⁺ and K⁺ ions in a molar ratio 2:1, similar to that in incubated slices. Since the potassium content of samples from the pooled medium was comparatively small, two larger samples of medium and two special standards with Na⁺ and K⁺ ions in a molar ratio 24:1, similar to that of the medium, were used for this measurement. The coefficient of variation for all experiments was 3.7% for sodium standards and 3.0% for potassium standards.

Chloride. Except for the determination of the blank, which was performed in quadruplicate, analyses of samples from each flask for chloride were carried out in duplicate by the method of Lowry, Roberts, Leiner, Wu & Farr (1954) on a larger scale: 0.5 ml. of 1.4 mM-AgNO₃ in 3 N-HNO₃ was added to 1 ml. of sample in a centrifuge tube, gently mixed and allowed to stand for 30 min. at room temperature. The tubes were then centrifuged at 3000 rev./min. for 20 min. and 0.2 ml. of each supernatant was added to 5.0 ml. of rhodanine reagent. After the mixtures had stood for 1 hr. the extinctions at 470 mµ were read in a spectrophotometer. The coefficient of variation for chloride standards of all experiments was 5.7%.

Inulin. This was determined by the method of Varon & McIlwain (1961). In the present experiments, however, rinsing slices in 0.3 M-glucose was found to increase the tissue blank for inulin tenfold and was one of the reasons why this method of terminating the experiment was not

used here. The coefficient of variation for inulin standards of all experiments was 2.8%.

Expression of results

The difference between the initial fresh wet wt. of the slice and its weight after incubation provided a measure of the additional fluid (of specific gravity assumed to be 1.0) taken up during incubation. Assuming that this additional fluid is extracellular and in equilibrium with the medium after 25 min. incubation (Varon & McIlwain, 1961), the quantity of each ion and of inulin contained was calculated from the measured concentrations of ions and inulin in the pooled medium. This quantity was subtracted from the total amount of each substance in each tissue extract to give a measure of the substance in terms of the initial fresh wet wt. of each slice. All quantities are therefore expressed as $\mu\text{equiv.}$ or $\mu\text{g./g.}$ initial wt. of tissue.

The inulin space, expressed as $\mu\text{l./g.}$ initial wt., was calculated by assuming the quantity of inulin/g. initial wt. to be entirely extracellular at a concentration equal to the measured inulin concentration of the pooled medium. Thus

Inulin space ($\mu\text{l./g.}$ initial wt.)

$$= \frac{\mu\text{g. of inulin/g. initial wt.}}{\mu\text{g. of inulin}/\mu\text{l. of external fluid}}$$

The non-inulin space was obtained by subtracting the inulin space from the total volume of the tissue, assumed to be $1000 \mu\text{l./g.}$ initial wt. The quantity of each ion contained in the inulin space was also calculated from the measured concentration of that ion in the pooled medium. This quantity was subtracted from the total amount/g. initial wt. to give a measure of the quantity of the ion in the non-inulin space. For comparison, the ions contained in the apparent chloride space and non-chloride space were also calculated by using similar assumptions.

RESULTS

Choice of calcium concentration. During a previous study of the calcium content of guinea-pig cerebral-cortical tissue, prepared and incubated under conditions selected for the present experiments, the sodium and potassium contents of the tissue were found to depend on the calcium concentration of the incubating medium (Lolley, 1963). Results from the analysis of 42 samples of cerebral cortex for sodium, potassium and calcium are presented in Fig. 1. This Figure, based on unpublished work by Dr R. N. Lolley, demonstrates that incubated slices with less than $2.5 \mu\text{moles}$ of calcium/g. initial wt. had progressively lower contents of potassium and progressively increased contents of sodium. Above $2.5 \mu\text{moles}$ of calcium/g. the potassium content remained constant, but tissue containing $4 \mu\text{moles}$ of calcium/g. and over showed a progressive increase in sodium content.

The incubated tissue thus approximated most closely to tissue sampled *in vivo* in its content of sodium and potassium salts when its calcium content

Fig. 1. Sodium content (a) and potassium content (b) of 42 slices of guinea-pig cerebral cortex prepared after removal of the brain from the skull, and incubated for 30 min. in bicarbonate-buffered glucose-containing media with concentration of calcium from 0 to 5 mM. The results were calculated on the basis of initial wet wt. and are shown as a function of tissue content of calcium. The lines have been fitted by eye.

was between 2 and $3 \mu\text{moles/g.}$, which, further, is the calcium content of the tissue *in vivo*. To obtain this content of calcium in tissue incubated *in vitro*, Lolley (1963) found that the incubating media needed to be 0.75 mM in calcium salts rather than 2.8 mM, the concentration of calcium that has customarily been used in incubation media for many isolated tissues.

In a series of initial experiments designed to compare the response to electrical stimulation and subsequent recovery of slices incubated in media of these two calcium concentrations, slices obtained from the same brains were equally distributed between media 0.75 and 2.8 mM with respect to calcium. Analysis for ion content and inulin was carried out after incubation for 30, 40 and 50 min. Half the number of slices incubated for 40 min. as well as half of those incubated for 50 min. were stimulated between 30 and 40 min. incubation. The average changes in concentration of sodium and potassium in the non-inulin space of these slices are shown in Fig. 2. Although the differences between slices incubated at the two calcium concentrations are not all statistically significant, there is a larger net change and a more complete return to the state before stimulation in slices incubated in 0.75 mM-calcium compared with those incubated in 2.8 mM-calcium. This is further emphasized by the values

calculated for the concentration gradients of ions between medium and the non-inulin space for these three conditions (Table 1). Over 200 slices were

subsequently incubated in media containing calcium at a concentration of 0.75 mM: the differences in ion content and reaction to stimulation observed here were verified.

In another series of experiments, 17 slices cut *in situ* were weighed initially, incubated 30 min. in media containing calcium at a concentration of 0.75 mM, and reweighed after repeated draining on a clean glass surface. The average increase in weight of the drained tissue as compared with initial wt. was 13.5 ± 6.3 mg./100 mg. initial wt. This compares with a value of 22 mg./100 mg. obtained by Bachelard *et al.* (1962) with tissue handled in the same way except for incubation in 2.8 mM-calcium salts, and suggests that the lower calcium concentration may have diminished the fluid uptake that occurs during incubation of cerebral-cortex slices. For these reasons, media containing calcium at a concentration of 0.75 mM were used in subsequent experiments.

Fluid compartments. During this investigation analytical results for over 200 individual slices were accumulated. A large variation was found in the size of the inulin and apparent chloride spaces (Table 2). A measure of a functioning extracellular space is important, especially for studies of sodium movements, and these results have been examined for factors, in addition to those described by Varon & McIlwain (1961), that might systematically affect estimates of the size of the extracellular space.

Tissue inulin spaces corrected for additional fluid were not found to be affected by speed of preparation of the tissue (range: 140–380 sec.), by duration of incubation (range: 25–90 min.), or by electrical stimulation. On the other hand, a significant correlation was found between the size of inulin spaces of two slices obtained from the same cerebral hemisphere ($r = +0.35$, $P < 0.01$, 99 pairs of slices). In addition, a zonal difference of approximately the same magnitude as that reported by Varon & McIlwain (1961) was found, the slices cut first from the outer convexity of the cortex having on an average inulin spaces $63 \mu\text{l./g.}$ smaller than those of slices cut second. The significance of this latter

Fig. 2. Changes in concentrations of sodium (a) and potassium (b) in the non-inulin space of cerebral-cortex slices, in response to 10 min. electrical stimulation after 30 min. preincubation, and in slices allowed to recover for 10 min. after termination of pulses. ○, ●, Unstimulated tissue; △, ▲, stimulated tissue. ○, △, Incubation in 0.75 mM-calcium; ●, ▲, in 2.8 mM-calcium. Each point represents the average of four slices. Standard deviations are indicated by vertical bars.

Table 1. Intracellular/extracellular concentration gradients for sodium and potassium of cerebral-cortex slices incubated in media with 0.75 mM- or 2.8 mM-calcium

The concentration ratios $[\text{Na}]_e/[\text{Na}]_i$ and $[\text{K}]_i/[\text{K}]_e$ express the computed distribution of the ions between the non-inulin space (internal) and the inulin space (external) of the slice, assuming that the latter has the composition of the external medium.

	Unstimulated	Stimulated	Recovered
Duration of incubation (min.) ...	30	40	50
Time of stimulation (min.) ...	—	30–40	30–40
$[\text{Na}]_e/[\text{Na}]_i$ ratio 0.75 mM-Calcium	3.4	1.7	3.2
2.8 mM-Calcium	2.7	1.8	2.3
$[\text{K}]_i/[\text{K}]_e$ ratio 0.75 mM-Calcium	18.4	10.2	16.7
2.8 mM-Calcium	15.7	9.5	14.4

difference was evaluated for the 99 pairs of slices by means of a *t* test for correlated measures and was found to be highly significant ($t = 9.24$, $P < 0.001$). Although first and second slices differ in cellular structure (Hillman & McIlwain, 1961), it may be assumed (see below) that, since the first-cut slice has only one cut surface, the increase in inulin space of a second slice is due mainly to the two cut surfaces. Subtracting this difference of $63 \mu\text{l./g.}$ from the average inulin space of first slices, a value of $258 \mu\text{l./g.}$ initial wt. is obtained for the inulin space of that portion of incubated tissue not affected by cutting. If the effect of slicing is localized, one cut therefore directly affects 6.3% of the tissue volume or 0.02 mm. in a slice 0.35 mm. thick.

As shown in Table 2, the size of the computed chloride spaces varies as much as that of the inulin spaces. The apparent chloride space is always significantly larger than the inulin space for particular slice ($r = +0.41$, $P < 0.001$, $n = 198$ slices), and when Gibson & McIlwain (1965) calculated tissue space on the basis that chloride was in electrochemical equilibrium with a membrane potential of

–60 mv an extracellular space of $254 \mu\text{l./g.}$ was obtained.

Ion content of incubated slices. Total sodium and potassium, their concentrations in the non-inulin space, and the ratio between concentration in the medium and that calculated for the non-inulin space are shown in Table 3 for slices incubated in 0.75 mM-calcium under three selected conditions. In judging the variation, it should be noted that the groups of slices are composed of equal, or nearly equal, numbers of first-cut and second-cut slices. Freshly cut slices do not differ systematically in ion content according to the number of cut surfaces, but second slices after incubation have systematically larger inulin spaces, a proportionally larger total sodium and chloride content, and less potassium than have first slices.

Twenty-two unstimulated first-cut slices had an average potassium content of $75 \mu\text{equiv./g.}$, and 22 second slices had $65.2 \mu\text{equiv./g.}$ By using the difference in volume between inulin spaces of first and second slices ($63 \mu\text{l./g.}$) as a measure of additional cellular damage attributable to the second cut surface, and the average value for concentration of potassium in the non-inulin space of these 44 slices (112.3 mM), a value of $7.1 \mu\text{equiv.}$ of potassium/g. of tissue is found corresponding to this volume difference. Thus a large part of the $9.9 \mu\text{equiv./g.}$ difference in potassium content between first and second slices may be attributed to depletion of the second slice at its additional cut surface, assuming the cut to have a localized effect.

Corresponding values for sodium show an actual increase of $7.3 \mu\text{equiv./g.}$ in second slices over first slices, which compares with the $6.7 \mu\text{equiv./g.}$ net gain in sodium computed for the two processes presumed to occur as a result of the second cut surface: $9.4 \mu\text{equiv.}$ of additional sodium/g. estimated for a second cut surface of $63 \mu\text{l./g.}$ in equilibrium with the

Table 2. *Inulin spaces and apparent chloride spaces of cerebral-cortex slices*

The values are averages $\pm$ S.D., with ranges in parentheses, for 99 slices cut first (with one cut surface) and 99 slices cut second (with two cut surfaces), expressed as $\mu\text{l./g.}$ initial wt. The slices were incubated for 30–55 min., and the different groups contained equal numbers of stimulated and unstimulated slices.

	First slices	Second slices
Inulin space	321 ± 83.2 (88–507)	382.9 ± 74.0 (175–565)
Apparent chloride space	466.2 ± 71.0 (282–627)	525.1 ± 69.5 (336–683)

Table 3. *Ion content of cerebral-cortex slices before, during and after electrical stimulation*

The Table gives values for (a) total sodium, potassium and chloride (in $\mu\text{equiv./g.}$ initial wt.), (b) concentrations of these ions in the non-inulin space (mM), and (c) ratios of external/internal sodium and chloride concentrations or internal/external potassium concentrations. Values are given as means $\pm$ S.D. The calcium concentration of the incubation media was 0.75 mM.

	Unstimulated	Stimulated	Recovered
Duration of incubation (min.) ...	30	40	50
Time of stimulation (min.) ...	—	30–40	30–40
No. of slices ...	11	13	6
Total Na	79.9 ± 12.0	103.4 ± 14.4	87.9 ± 8.1
[Na] in non-inulin space	39.5 ± 11.4	82.8 ± 16.7	49.9 ± 9.5
$[\text{Na}]_o/[\text{Na}]_i$ ratio	4.0 ± 1.3	1.9 ± 0.3	3.2 ± 0.8
Total K	72.5 ± 5.4	49.9 ± 5.2	67.0 ± 6.5
[K] in non-inulin space	114.2 ± 18.2	69.6 ± 8.6	103.6 ± 12.7
$[\text{K}]_i/[\text{K}]_o$ ratio	18.9 ± 2.9	11.3 ± 2.0	16.3 ± 1.9
Total Cl	61.3 ± 12.8	61.4 ± 10.2	69.3 ± 10.3
[Cl] in non-inulin space	22.8 ± 11.8	32.1 ± 7.2	29.4 ± 1.0
$[\text{Cl}]_o/[\text{Cl}]_i$ ratio	9.1 ± 9.9	4.2 ± 1.2	5.2 ± 2.2

medium, minus the loss of $2.7 \mu\text{equiv.}$ of sodium/g. from the non-inulin space of damaged cells. The increase found in chloride in second over first slices is $7.2 \mu\text{equiv./g.}$ The net estimate for chloride gain, computed as above, is $6.9 \mu\text{equiv./g.}$

Thus systematic differences between first and second slices contribute to the large standard deviations of Table 3 for total ions. An analysis of variance of the results shown in Table 3 reveals that position of the slice contributes 30–40% of the total variance in potassium content, 30% in chloride content and 10% in sodium content. The F values for unstimulated tissue are: sodium, 7.78; potassium, 36.25; chloride, 36.1. All these are highly significant values. The effect with sodium is smaller than that with chloride and potassium, presumably because the concentration gradient of sodium between medium and incubated tissue is smaller. Position of the slice contributed about 30–40% to the total variance of potassium of the non-inulin space also, this proportion remaining about the same during stimulation and recovery. However, there is no indication that the quantity of sodium in the non-inulin space was affected, as might be expected from the relatively low and highly variable concentration of sodium in the non-inulin space.

It is nevertheless clear that incubated slices cut second have decreased potassium and increased sodium and chloride, and that the quantities are proportional to the increase in the volume of the inulin space. Values for the fraction of sodium, potassium and chloride contents of the incubated tissue not directly affected by cutting have been derived from the above estimates and are compared with results for fresh and uncorrected incubated tissue in Table 4.

Ion content of stimulated slices. The time-course of the changes between the points selected for display in Table 3 are shown for sodium in Fig. 3 and for potassium in Fig. 4. To obtain these curves eight

Table 4. *Ion content of incubated and of fresh guinea-pig cerebral-cortex tissue*

On the basis of the estimated volume of the layer damaged by cutting ($63 \mu\text{l./g.}$ of tissue), analytical results for top slices with one cut surface (row *a*) have been corrected to represent ion content of incubated tissue not affected by cutting (row *b*). Row (*c*) gives the ion content found in fresh tissue.

	Content ($\mu\text{equiv./g. initial wt.}$)		
	Sodium	Chloride	Potassium
(<i>a</i>) Incubated top slice	81.2	64.5	75.1
(<i>b</i>) Incubated top slice (corrected)	74.5	57.6	82.2
(<i>c</i>) Fresh tissue	44.6	41.0	96.0
Difference (<i>b</i>)–(<i>c</i>)	(+29.9)	(+16.6)	(–13.8)

Fig. 3. Time-course of changes in the sodium of the non-inulin-space of cerebral-cortex slices, preincubated for 30 min., in response to 10 min. electrical stimulation and during 10 min. recovery after cessation of stimulation. The glucose-bicarbonate incubation medium contained calcium at a concentration of 0.75 mM . Each point represents the average of six to eight slices, except the value for the unstimulated group (zero time), which comprised 45 slices. Vertical bars indicate standard deviations.

Fig. 4. Time-course of changes in the potassium of the non-inulin space of cerebral-cortex slices, preincubated for 30 min., in response to 10 min. electrical stimulation and during 10 min. recovery after cessation of stimulation. The glucose-bicarbonate incubation medium contained calcium at a concentration of 0.75 mM . Each point represents the average of six to eight slices, except the value for the unstimulated group (zero time), which comprised 45 slices; vertical bars indicate standard deviations.

slices from two guinea pigs were used in each experiment, one of the slices from each hemisphere being stimulated and the other being used as an unstimulated control incubated for an identical time-interval. Four consecutive points on the graphs were thus covered in one experiment. By applying stimulation to top and second slices alternately and obtaining results for each point from equal numbers of each slice category, systematic errors of this sort were eliminated.

The ion content of unstimulated slices did not change during incubation for up to 90 min. (Table 5). Stimulation caused substantial gain in sodium and loss of potassium. The resulting concentrations remained stable for periods of stimulation as long as 60 min. (not shown in Figs. 3 and 4). The time-courses of the net changes in sodium and potassium suggest that reaction to stimulation commenced rapidly and was completed in about 6 min.; efflux and influx presumably balance each other when stimulation is continued beyond this point (Keese & Wallgren, 1965). An estimate of the initial rate of net entry of sodium from the slope of the curve in Fig. 3 gives a value of $460 \mu\text{equiv./g. initial wt./hr.}$ The corresponding value for loss of potassium is $480 \mu\text{equiv./g./hr.}$ These are, within the errors of measurement, identical values.

Within 10 min. after termination of pulses, recovery was complete or nearly complete, the values being statistically indistinguishable from those before stimulation. The initial rate of net change during recovery is about $160 \mu\text{equiv./g./hr.}$ for sodium and $230 \mu\text{equiv./g./hr.}$ for potassium. It thus appears from these estimates that the rate of net change on recovery is slower than that during the initial response to the onset of stimulation.

The rates of net sodium and potassium movements are very similar, and the general time-courses closely mirror each other. However, on stimulation the net content of sodium increased more than that of potassium decreased, and sodium concentration returned more slowly to its initial value on termination of stimulation. Thus the sum of sodium and potassium of the non-inulin space increased on stimulation, and on termination of pulses this quantity increased still further (Fig. 5), presumably reflecting the faster net entry of potassium than net loss of sodium during the early phase of recovery. These differences approach statistical significance ($0.05 < P < 0.1$ for largest difference) and a comparable association was indicated by the following observations on changes in chloride content during electrical stimulation.

Although the total chloride content of the tissue changed little during stimulation and on recovery, the chloride of the non-inulin space changed, as shown in Fig. 5, in a manner similar in time-course and amount of total change to the changes described

Fig. 5. Changes in the sodium plus potassium content (a) and in the chloride content (b) of the non-inulin space in cerebral-cortex slices in response to 10 min. electrical stimulation and during 10 min. recovery after cessation of stimulation. Each point gives the average of six to eight slices, except the value for unstimulated slices (zero time), which is based on 45 slices. Standard deviations are indicated by vertical bars.

above for the sum of sodium and potassium in the non-inulin space. On stimulation there was an increase in the chloride of the non-inulin space, and this rose further during the early phase of recovery and returned close to the pre-stimulation concentration within 6 min. after termination of pulses. A close relation between chloride and cations of the non-inulin space is also indicated by the statistically highly significant positive correlation ($r = +0.8$, $P < 0.01$, $n = 46$) between the widely varying sodium and chloride of the non-inulin space of unstimulated slices.

DISCUSSION

Ion content of incubated cerebral-cortex slices. One of the features of cerebral tissues most difficult to maintain *in vitro* has been the relatively low sodium content and high potassium content that exist *in vivo* (Table 4). The processes that have been suggested to account for these differences in ion composition between fresh and incubated cerebral cortex have included mechanical damage to cells in the cut surface layer leading in particular to potassium depletion (Pappius & Elliott, 1956b), exchange of tissue potassium for sodium in the incubating fluid

and net uptake of fluid containing sodium and chloride (Leaf, 1956; Bachelard *et al.* 1962).

The fluid changes associated with preparation and incubation of cerebral-cortex slices have been examined in detail by Varon & McIlwain (1961), who divided the additional fluid uptake into three categories: (1) fluid that adheres to slices on immersion into and removal from medium; (2) irreversible swelling that occurs if the slices are placed even for a few seconds in inadequately oxygenated media, the swelling being intracellular in the sense that it is not associated with the uptake of inulin; (3) a slow increase in the size of the inulin space, after placing slices in adequate metabolic conditions, that is extracellular in the sense that it is not associated with a reduction in the size of the non-inulin space.

Table 5. *Ion content of cerebral-cortex slices after different periods of incubation.*

Values are given as means $\pm$ s.d.

Duration of incubation (min.)	No. of slices	Total content (μ equiv./g. initial wt.)		
		Sodium	Potassium	Chloride
30	46	84.6 $\pm$ 11.1	70.5 $\pm$ 7.9	67.8 $\pm$ 6.8
40	32	89.9 $\pm$ 8.9	67.8 $\pm$ 10.1	72.2 $\pm$ 11.2
50	17	89.0 $\pm$ 10.1	68.5 $\pm$ 8.9	68.4 $\pm$ 10.8
90	8	93.1 $\pm$ 8.2	68.3 $\pm$ 7.9	74.7 $\pm$ 6.2

Bachelard *et al.* (1962), by rapid preparation and the use of adequately oxygenated media, sought to minimize the irreversible fluid changes described in category (2) above. They were successful in obtaining slices in which a net extrusion of sodium during incubation could be demonstrated for the first time, and total fluid uptake during 30 min. incubation at 37° was limited to 22% of initial tissue, compared with values of 26–30% reported by Varon & McIlwain (1961) and of 36–41% reported by Leaf (1956), Pappius & Elliott (1956a) and Pappius, Klatzo & Elliott (1962). In the present investigation, in which an external calcium concentration of 0.75 mM was used, the average total fluid uptake of 17 slices, drained as in the experiments of the above authors, was 13.5% after 30 min. incubation. Approx. 10% of this increase in weight may be attributed to category (1), i.e. adhering fluid resulting from immersion into and removal of a dry-cut slice from saline medium (Varon & McIlwain, 1961), leaving a fluid increase of only about 3% of the tissue wt. This small increase is of the magnitude attributable to category (3), which occurred in the extracellular space during incubation at the rate of 0.1 μ l./100 mg./min. (Varon & McIlwain, 1961).

Thus cerebral-cortex slices prepared and incubated as described in the present investigation reach a relatively steady state with respect to total fluid content within 30 min. incubation, and for the

Table 6. *Changes in ion and fluid content of incubated cerebral-cortex slices, prepared and incubated under different experimental conditions, compared with the ion and fluid content of fresh tissue*

All slices were cut without additional lubricating fluid. The column marked 'Brain' designates whether the slices were cut after the brain had been 'removed' from the skull or while the brain remained *in situ* in the skull. All slices were incubated in glucose-bicarbonate media in equilibrium with O₂ + CO₂ (95:5), except those reported by Leaf (1956), which were incubated in a medium containing only NaCl (160 mM) and KCl (5 mM).

Reference	Species	Brain	Pertinent experimental conditions			Changes in ion content (μ equiv./g. initial wt.)				Changes in fluid content (%)
			Slice thickness (mm.)	Incuba- tion temp.	External calcium (mm)	Sodium	Chloride	Sodium— chloride		
								Potassium		
Leaf (1956)	Rat	Removed	0.3	0°	0	+108	+67	+41	—58	+39
Terner, Eggleston & Krebs (1950)	Guinea pig	Removed	0.4	40	2.6	—	—	—	—44	—
Pappius & Elliott (1956a,b)	Rat	Removed	0.5	38	1.3	+56	—	—	—73	+41
Varon & McIlwain (1961)	Guinea pig	Removed	0.3	37	2.8	+99	+67.5	+31.5	—37.5	+28
Bachelard <i>et al.</i> (1962)	Guinea pig	Removed	0.3	37	2.8	+79	+39	+40	—39	+27
Bachelard <i>et al.</i> (1962)	Guinea pig	<i>in situ</i>	0.3	37	2.8	+45	+34.5	+10.5	—35	+22
Present study (un- corrected values)	Guinea pig	<i>in situ</i>	0.35	37	0.75	+40	+27	+13	—25.5	+13.5
Present study (cor- rected for cut surfaces)	Guinea pig	<i>in situ</i>	0.35	37	0.75	+30	+17	+13	—14	—

most part the distribution of this fluid resembles that of freshly cut cerebral-cortex slices. In addition, slices so prepared and incubated reach a steady state with respect to their contents of sodium, potassium and chloride (Table 5), and this steady state more closely resembles that existing *in vivo* than has been reported previously for mammalian cerebral-cortex slices incubated in physiological saline media (Table 6).

However, even after allowance is made for ions contained in adhering fluid and also for changes in ion content resulting from the cut surfaces in the slice (Table 4), there still remains an excess of 30 μ equiv. of sodium/g. and 17 μ equiv. of chloride/g. and a deficit of 14 μ equiv. of potassium/g. in the incubated slices compared with values obtained from freshly cut cerebral-cortex slices. Changes that have not resulted in the desired improvement of ion composition of incubated slices without interfering with other aspects of the tissue composition and metabolism are listed in Table 7.

Ion content and membrane potentials. The closeness of the sodium, potassium and chloride contents of the present slices to values obtaining *in situ* is in many ways remarkable, when it is recalled that the permeability of neural tissues to these ions is inherently variable. Thus the nerve impulse is pictured to result from a sequence of changes in permeability to, preponderantly, Na^+ and K^+ ions during which their relative permeabilities change over a 100-fold range. By micro-pipette electrodes, a mean resting membrane potential, E_m , of -60 mv has been observed in cerebral-cortical slices prepared and incubated under the present conditions (Hillman & McIlwain, 1961; Gibson & McIlwain, 1965). Accepting this potential and the present tissue analysis as referring to the same cellular entities (Gibson & McIlwain, 1965), the observed potential is markedly less negative than the equilibrium potential for $[\text{K}^+]/[\text{K}^+]_o$ in the unstimulated tissue of Tables 1 or 3. Presumably, therefore, the opposing sodium gradient is partly effective.

Table 7. *Substances added to incubation media that have not improved ion content of incubated cerebral-cortex slices without interfering with other aspects of tissue composition and metabolism*

References: (1) Ashford & Dixon (1935); (2) Bachelard *et al.* (1962); (3) Dickens & Greville (1935); (4) Gore & McIlwain (1952); (5) Hillman & McIlwain (1961); (6) Joanny & Hillman (1963); (7) Krebs, Eggleston & Turner (1951); (8) Pappius & Elliott (1956a); (9) Pappius, Rosenfeld, Johnson & Elliott (1958); (10) Turner *et al.* (1950); (11) Tower (1959); (12) Varon & McIlwain (1961).

Substance added	Summary of known effects, if any, on brain slices	Reference(s)
Fructose (20 mM)	Increased Na and K	(6)
Succinate (20 mM)	Increased Na	(6)
Oxaloacetate (30 mM)	Increased Na and K	(6)
Pyruvate (5 mM, 20 mM)	Increased Na and K	(6), (8)
Fumarate (5 mM, 20 mM)	Increased Na	(6), (8)
L-Glutamate (5 mM, 10 mM, 20 mM)	Increased Na and K	(6), (8), (10)
	Increased intracellular space	(8)
	Decreased E_m	(5)
Ascorbate (1 mM)	No improvement in Na or K	(2)
Creatine (1 mM)	No improvement in Na or K	(2)
Adenosine (1 mM)	No improvement in Na or K	(2)
Guanosine (1 mM)	No improvement in Na or K	(2)
Gangliosides (0.2–0.3 mg./ml.)	Increased Na	(2)
Plasma fraction IV-4 (10 mg./ml.)	Increased Na	(2)
Homologous serum	No improvement in Na or K	(2), (7), (8)
	Increased non-SCN space	(8)
Protamine (0.15 mg./ml.)	Decreased intracellular and extracellular space	(12)
Sucrose (10%)	Decreased non-SCN space	(8)
	Decreased oxygen uptake	(8)
Polyvinylpyrrolidone (7%)	Increased non-SCN space	(8)
	Decreased oxygen uptake	(8)
Sodium (less than 100 mM)	Decreased Na and K	(2), (9)
	Decreased E_m and metabolic response	(5)
Potassium (27–40 mM)	Increased K and decreased Na	(11), (8)
	Increased non-SCN space	(8)
	Increased respiration	(1), (3)
	Decreased phosphocreatine	(4)
	Decreased E_m	(5)

Applying the Nernst equation (Hodgkin, 1958) in the form:

$$E_m = \frac{RT}{F} \ln \frac{[K]_e + b[Na]_e}{[K]_i + b[Na]_i} \quad (1)$$

where subscripts e and i designate extra- and intracellular respectively (activities are taken as proportional to concentrations) and b gives the apparent permeability of sodium relative to that of potassium, it was concluded (Gibson & McIlwain, 1965) that potential and composition in the isolated unstimulated tissue could be reconciled when b approximated to 0.05. Among neural tissues these relative permeabilities are best understood in the squid axon, and here b at rest can be 0.01–0.08, changing during excitation to a value that momentarily may approximate to 30 (Hodgkin, 1959; Baker, Hodgkin & Shaw, 1962). This feature of the resting tissue is thus well maintained *in vitro*.

The results in Table 3 may also be examined to judge the extent to which chloride distribution is in electrochemical equilibrium with an observed membrane potential of -60 mv. This potential would correspond to a value of 9.3 for the $[Cl^-]_e/[Cl^-]_i$ ratio. Observed ratios in the non-inulin space of tissues before stimulation are similar to this value (Table 3). Thus an active assimilation of chloride by the resting tissue such as found by Keynes (1963) in squid giant axons is not supported, although after excitation the value is lower (Table 3; see Gibson & McIlwain, 1965).

Changes in ion content during electrical stimulation and subsequent recovery. It is impressive to see from Fig. 3 and Table 3 that a few minutes of electrical stimulation can double the net sodium content of the non-inulin space, and that on cessation of electrical pulses sodium is rapidly extruded against a concentration gradient. Such results lend support to the view that an increased rate of active ion transport is largely responsible for the increased energy utilization during electrical stimulation of isolated cerebral-cortical tissue (McIlwain, 1963).

In the steady state during prolonged electrical stimulation turnover of both sodium (Keesey & Wallgren, 1965) and potassium (Cummins & McIlwain, 1961), as measured by radioactive isotopes, is increased. However, the present results do not provide evidence about whether electrical events or changes in ionic concentrations at the cell membrane provide the stimulus for increased rates of transport. Evidence from other neural preparations suggests that a rise in intracellular sodium stimulates an increased rate of sodium extrusion (Hodgkin & Keynes, 1955, 1956; Eccles, 1957); from the present study the change from 40 mM to 80 mM in the concentration of sodium in the non-inulin space is in the range known to stimulate the Na^+ - and K^+ -sensitive adenosine-triphosphatase activity of cerebral microsomes (Deul & McIlwain, 1961; Schwartz,

Bachelard & McIlwain, 1962). However, the time-course of the changes in ion content during onset and termination of electrical stimulation (Figs. 3 and 4) correspond very well to the changes in intracellular membrane potential observed by Hillman *et al.* (1963) during the same time-intervals.

When membrane potential becomes less negative in a system to which chloride is permeable, chloride entry is to be expected. Fig. 5 and Table 3 show such entry when the tissue is depolarized by electrical stimulation, followed by partial extrusion within 5 min. of cessation of pulses. However, besides the large variation in chloride concentration in the non-inulin space, the sluggishness of inulin movements (Varon & McIlwain, 1961) makes these conclusions tentative only, because rapid changes in the size of space such as may occur during a few minutes of electrical stimulation may not be reflected immediately by changes in inulin content.

Ion content and external calcium concentration. When the external calcium concentration is lowered the cerebral-cortex slices, after incubation, not only have a steady fluid and ion content more closely approximating to those existing *in vivo*, but also respond to electrical stimulation with an initial rate of net sodium gain and potassium loss of 460–480 μ equiv./g./hr. This value is about twice that of 180–240 μ equiv./g./hr. reported by Bachelard *et al.* (1962) with an external calcium concentration of 2.8 mM. Further, after cessation of pulses in an external calcium concentration of 2.8 mM, net movements of sodium and potassium were greatly slowed down when only one-third to one-half of the ions displaced had been recovered (Bachelard *et al.* 1962). In an external calcium concentration of 0.75 mM recovery of initial sodium (Fig. 3) and potassium contents (Fig. 4) was complete, or nearly complete, 10 min. after electrical stimulation had been stopped.

The mechanism of the various effects of lowered external calcium concentration on fluid and ion content of incubated cerebral-cortex slices and their response to the onset and termination of electrical stimulation has not been specifically investigated in the present study. However, Lolley (1963) found that the calcium content of incubated cerebral tissue more closely resembled that found in fresh tissue (2–3 μ moles/g.) when the external calcium concentration of glucose-bicarbonate media was 0.75 mM rather than 2.8 mM, the latter value having been based originally on the total calcium concentration found in rat and guinea-pig sera (Krebs, 1950; Lolley, 1963). This is not unexpected, considering that much calcium in blood plasma is bound, and that the total calcium concentration in cerebrospinal fluid of the guinea pig was found to be 1.6 mM, at least 10% of which was bound to protein (Lolley, 1963).

More unexpected, perhaps is the improvement in the tissue concentration of the univalent cations at this particular calcium concentration (Fig. 1). Calcium is usually considered to be a membrane 'stabilizer' (Shanes, 1958), and decreased external calcium generally leads to increased movement of sodium into some cells, e.g. into human erythrocytes (Maizels, 1961), across frog skin (Curran & Gill, 1962), into rat diaphragm muscle (Harris, 1960) and into squid axon (Adelman & Moore, 1961). This behaviour of sodium is also demonstrated in cerebral-cortex slices at external calcium concentrations below 0.75 mM (Fig. 1) and by the approximate doubling of the rate of net entry of sodium into cerebral-cortex slices on electrical stimulation when the external calcium concentration is 0.75 mM (Fig. 3) rather than 2.8 mM (Bachelard *et al.* 1962). However, in other instances lowering the external calcium concentration also appears to facilitate movement out of cells, as now seen in the more complete recovery of initial sodium concentrations of cerebral-cortex slices after electrical stimulation in the lower external calcium concentration (Figs. 2 and 3). This effect has also been found in other biological systems: in rat small intestine, for instance, a calcium concentration in the external fluid below 1 mM increased sodium efflux, whereas at higher concentrations sodium efflux was inhibited (Dumont, Curran & Solomon, 1960). In erythrocyte 'ghosts' a decreased calcium/ATP ratio proportionately increased sodium efflux (J. Hoffman & H. Ryan, quoted by Hoffman, 1960), and in liver decreased external calcium decreased sodium content and swelling while increasing phosphoprotein turnover (Judah & Ahmed, 1962). In toad muscle external calcium depletion reversibly accelerated a slow phase of sodium efflux (Simon, Muller & Satchell, 1962).

Decreased external calcium may facilitate movement of sodium out of cells by one or more of several possible mechanisms, including decreased competition between calcium and sodium for carrier or adsorption sites concerned with transport. The result in Table 1 together with the composition of the medium may be used to calculate values of b in eqn. (1), if some assumptions are made about membrane potential. Thus Gibson & McIlwain (1965) found no significant difference between membrane potentials of tissues incubated in 0.75 mM- and 2.6 mM-calcium, though variation was large. However, by taking the mean membrane potential in each case as -60 mV, the relative sodium/potassium permeabilities, b , are found to be 0.039 at the lower and 0.029 at the higher calcium concentration. On electrical stimulation and assuming complete depolarization of the tissue in each case (see Hillman *et al.* 1963, for the extrapolation involved), b values are 0.85 and 0.76, again lower with the higher cal-

cium concentration. To ascribe these differences to any one of the several factors involved requires examination of individual ion movements, and to this the next paper (Keesey & Wallgren (1965) contributes.

We express our appreciation to Dr Richard Lolley for permission to include Fig. 1, and to Mr Naren Banik and Miss Brenda Aylett for excellent technical assistance. Special thanks are due to Joan Willis Keesey for writing the programme to process our data and to the IBM Endowed Research Time Scheme for time on the IBM 7090 computer. H. W. was on leave of absence from the Research Laboratories of the State Alcohol Monopoly (Alko), Helsinki, Finland, as a British Council Scholar, with partial support from Alko and the Finnish State Scientific Council. J. K. was supported during this investigation by a North Atlantic Treaty Organization (NATO) Postdoctoral Fellowship in Science from the National Science Foundation (U.S.A.).

REFERENCES

- Adelman, W. J. & Moore, J. W. (1961). *J. gen. Physiol.* **45**, 93.
- Ashford, C. A. & Dixon, K. C. (1935). *Biochem. J.* **29**, 157.
- Bachelard, H. S., Campbell, W. J. & McIlwain, H. (1962). *Biochem. J.* **84**, 225.
- Baker, R. F., Hodgkin, A. L. & Shaw, T. I. (1962). *J. Physiol.* **164**, 355.
- Cummins, J. T. & McIlwain, H. (1961). *Biochem. J.* **79**, 330.
- Curran, P. F. & Gill, J. R., jun. (1962). *J. gen. Physiol.* **45**, 625.
- Deul, D. H. & McIlwain, H. (1961). *J. Neurochem.* **8**, 246.
- Dickens, F. & Greville, G. D. (1935). *Biochem. J.* **29**, 1468.
- Dumont, P. A., Curran, P. F. & Solomon, A. K. (1960). *J. gen. Physiol.* **43**, 1119.
- Eccles, J. C. (1957). *The Physiology of Nerve Cells*, p. 71. London: Oxford University Press.
- Gibson, I. M. & McIlwain, H. (1965). *J. Physiol.* (in the Press).
- Gore, M. B. R. & McIlwain, H. (1952). *J. Physiol.* **117**, 471.
- Gornall, A. G., Bardawill, C. J. & David, M. M. (1949). *J. biol. Chem.* **177**, 751.
- Harris, E. J. (1960). *Transport and Accumulation in Biological Systems*, 2nd ed., p. 165. London: Butterworths.
- Hillman, H. H., Campbell, W. J. & McIlwain, H. (1963). *J. Neurochem.* **10**, 325.
- Hillman, H. H. & McIlwain, H. (1961). *J. Physiol.* **157**, 263.
- Hodgkin, A. L. (1958). *Proc. Roy. Soc. B*, **148**, 1.
- Hodgkin, A. L. & Keynes, R. D. (1955). *J. Physiol.* **128**, 28.
- Hodgkin, A. L. & Keynes, R. D. (1956). *J. Physiol.* **131**, 592.
- Hoffman, J. (1960). In *Ciba Found. Study Group no. 5: Regulation of the Inorganic Ion Content of Cells*, p. 75. Ed. by Wolstenholme, G. E. W. & O'Connor, C. H. London: J. and A. Churchill Ltd.
- Joanny, P. & Hillman, H. H. (1963). *J. Neurochem.* **10**, 655.
- Judah, J. D. & Ahmed, K. (1962). *Nature, Lond.*, **194**, 382.
- Keesey, J. C. & Wallgren, H. (1965). *Biochem. J.* **95**, 301.
- Keynes, R. D. (1963). *J. Physiol.* **169**, 690.
- Krebs, H. A. (1950). *Biochim. biophys. Acta*, **4**, 249.
- Krebs, H. A., Eggleston, J. V. & Terner, C. (1951). *Biochem. J.* **48**, 530.

- Leaf, A. (1956). *Biochem. J.* **62**, 241.
- Lolley, R. N. (1963). *J. Neurochem.* **10**, 665.
- Lolley, R. N. & McIlwain, H. (1964) *Biochem. J.* **93**, 12 r.
- Lowry, O. H., Roberts, N. R., Leiner, K. Y., Wu, M.-L. & Farr, A. L. (1954). *J. biol. Chem.* **207**, 1.
- McIlwain, H. (1954). *J. Physiol.* **124**, 117.
- McIlwain, H. (1963). *Chemical Exploration of the Brain, a Study of Cerebral Excitability and Ion Movement*, p. 43. Amsterdam: Elsevier Publishing Co.
- McIlwain, H. & Joanny, P. (1963). *J. Neurochem.* **10**, 313.
- McIlwain, H. & Rodnight, R. (1962). *Practical Neurochemistry*, p. 111. London: J. and A. Churchill Ltd.
- Maizels, M. (1961). In *Membrane Transport and Metabolism*, p. 256. Ed. by Kleinzeller, A. & Kotyk, A. London: Academic Press (Inc.) Ltd.
- Pappius, H. M. & Elliott, K. A. C. (1956a). *Canad. J. Biochem. Physiol.* **34**, 1007.
- Pappius, H. M. & Elliott, K. A. C. (1956b). *Canad. J. Biochem. Physiol.* **34**, 1053.
- Pappius, H. M., Klatzo, I. & Elliott, K. A. C. (1962). *Canad. J. Biochem. Physiol.* **40**, 885.
- Pappius, H. M., Rosenfeld, M., Johnson, D. M. & Elliott, K. A. C. (1958). *Canad. J. Biochem. Physiol.* **36**, 217.
- Schwartz, A., Bachelard, H. S. & McIlwain, H. (1962). *Biochem. J.* **84**, 626.
- Shanes, A. M. (1958). *Pharmacol. Rev.* **10**, 59.
- Simon, S. E., Muller, M. H. & Satchell, D. G. (1962). *Biochim. biophys. Acta*, **60**, 126.
- Terner, C., Eggleston, L. V. & Krebs, H. A. (1950). *Biochem. J.* **47**, 139.
- Tower, D. B. (1959). In *Biochemistry of the Central Nervous System*, pp. 239, 240. Ed. by Brücke, F. London: Pergamon Press Ltd.
- Varon, S. & McIlwain, H. (1961). *J. Neurochem.* **8**, 262.