systems and intracellular elements directly to electrodes. Earlier observations of effects of electrical currents and pulses of various types afford examples of inhibition and acceleration recognized as probably secondary to electrolysis or to metals from electrodes (see Duclaux, 1899; Oppenheimer, 1910). Effects of applied pulses were therefore examined on two enzyme systems relevant to the control of respiratory level and phosphate transfer. Table 2 shows that creatine phosphokinase was markedly inhibited by even brief exposures to pulses employed in preceding experiments, from gold or silver electrodes. Inhibition with molybdenum electrodes was much less, but still observable (Table 2). This contrasts markedly with properties of these metals as electrodes with slices, when each afforded the same considerable increase in respiration and glycolysis (Table 1).

Liberation of inorganic phosphate from adenosine triphosphate by cerebral 'homogenates' under Gore's (1951) conditions was unaffected by pulses described in Table 2, from molybdenum electrodes.

DISCUSSION AND SUMMARY

It is to be expected that some changes immediately linking applied electrical pulses and reactions in cell-containing systems, will be reproduced in cell-free systems; and also that mitochondria may exhibit certain analogous phenomena depending on permeability barriers which nevertheless need not be the basis for reaction of cell-containing tissues. Present experiments however show pulses adequate or even a little excessive for maximal stimulation of respiration in cell-containing cerebral tissues to

cause with suitable electrodes little or no change in oxygen uptake in presence of cerebral suspensions and mitochondrial preparations. Concomitant phosphorylation showed similarly little change. The changes observed in some cases may be secondary to deleterious effects induced by the pulses in sensitive intracellular systems, and of these an example has been encountered in creatine phosphokinase. Inhibition of the phosphokinase was greatly dependent on the metals used for electrodes. Comparable dependence on the electrode material was not found in working with cell-containing preparations of cerebral tissues.

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Applied Electrical Pulses and the Ammonia and Acetylcholine of Isolated Cerebral Cortex Slices

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Acetylcholine changes in the brain *in vivo* seem to be associated with its electrical activity (see Feldberg, 1945a). In particular it has been demonstrated that *in vivo* electrical stimulation diminishes considerably the total extractable acetylcholine of

brain; further that the acetylcholine returns close to the normal level before convulsions start; during their progress the level is again lowered (Richter & Crossland, 1949). Electrical stimulation, or the administration of picrotoxin, have also been found to increase the free ammonia of the brain (Richter & Dawson, 1948) and this increase occurs before the development of convulsions.

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If similar metabolic changes could be demonstrated to occur in isolated portions of the central nervous system on the application of electrical pulses, then investigations at this level could be extended to include a study of the effect of various agents on the changes. Comparative observations on normal and abnormal cerebral tissues would also be possible. This paper gives an account of experiments with cerebral cortex slices in which ammonia and acetylcholine changes following the application of electrical pulses have been studied. The techniques developed by McIlwain (1951) for the application of electrical pulses to isolated parts of the central nervous system have been employed.

EXPERIMENTAL

Slice preparation, incubation, and the application of electrical pulses. Slices were incubated in phosphate-glucose saline containing 0.127 M-NaCl, 0.0051 M-KCl, 0.00127 M-KH₂PO₄, 0.00127 M-MgSO₄, 0.01 M-Na₂HPO₄ (brought to pH 7.4 with HCl), 0.00275 M-CaCl₂ and 0.01 M glucose, saturated with O₂ at room temperature.

Cerebral cortex slices were cut parallel to the outer surface, trimmed to fit the tissue-holding electrodes and weighed, as described by McIlwain (1951). In the ammonia experiments slices weighed 80–140 mg. and in individual experiments pairs were matched to within 15 mg. (5 mg. for acetylcholine). To eliminate from consideration, where possible, variations in metabolism which might be attributable to the different depths from the surface of the cortex at which slices were cut, slices which received pulses and those which did not were matched, in the individual experiments, with respect to the order in which they were cut. The first cut from the surface of the cortex is described as an 'outer', the second as an 'inner' slice. Except where stated otherwise, all slices were fitted into the type D tissue-holding electrodes and these then fixed in 5.0 ml. of saline contained in type A¹ electrode manometer vessels (McIlwain, 1951).

Incubations were under O₂ at 37°. The respiratory increase on the application of electrical pulses (McIlwain, 1951) was measured. Electrical pulses were applied as alternating condenser pulses (Ayres & McIlwain, 1953) at 100/sec., time constant 0.3–0.4 msec., and peak potential 15–17 v.

Ammonia estimations. Ammonia was estimated by micro-diffusion analysis (Conway, 1947) using 0.001 or 0.0005 N-HCl. The saline and trichloroacetic acid (TCA) used for denaturing the slices were made with ammonia-free glass-distilled water. Frequent checks on the reliability and accuracy of the method were made using a standard (NH₄)₂SO₄ solution. For the determination of ammonia in incubated cerebral cortex slices 3 or 4 were pooled (total fresh wt. 110–150 mg.), dropped with any adhering saline into 1.2 ml. ice-cold 10% (w/v) TCA in a tared centrifuge tube, immediately brought into suspension with the use of a close-fitting glass pestle, and the tube was re-weighed. Tubes were centrifuged at 0° in a high-speed refrigerated centrifuge and the supernatants retained for ammonia analysis. To avoid hydrolysis of tissue glutamine in the TCA, ammonia estimations on 1.0 ml. samples were carried out as soon as possible, and awaiting estimation the solutions were maintained at –15° (see Richter & Dawson, 1948).

Acetylcholine assays. Acetylcholine was assayed routinely by observing the contractions of frog rectus abdominis muscle, following essentially the procedure employed by Quastel, Tennenbaum & Wheatley (1936) with leech muscle. A 3 ml. capacity muscle bath was used, cooled by circulating cold water. The frog-Ringer solution contained 9.0 g. NaCl, 0.42 g. KCl, 0.24 g. CaCl₂, 0.15 g. NaHCO₃ and 13.5 g. glucose made up to 1350 ml. in glass-distilled water. In the muscle bath it was continuously aerated. After allowing the muscle to relax for at least 1 hr. in non-eserinized Ringer under a tension of about 1 g., Ringer containing 0.001% eserine sulphate was added 1 hr. before acetylcholine assays were started. Eserine sulphate (0.001%) was employed for the standards, for the solutions undergoing assay and during the relaxation periods. (Some slices were incubated in media containing 0.01% eserine sulphate and the media assayed using a frog muscle where the same concentration of eserine was employed throughout. It was found, however, that this relatively high concentration of eserine greatly diminished the contractile ability of the muscle and routinely 0.001% eserine sulphate was used.) The frog muscle was weighted only by the attached lever after the initial 2 hr. relaxation period.

Assays were conducted on frog muscle usually within the range 0.05–0.10 µg. acetylcholine/2 ml., in which estimation was possible to within 0.005 µg. acetylcholine/2 ml. The error was therefore 5–10%. The rectus abdominis muscle varies greatly from one frog to another (*Rana temporaria* males were used) in its ability to respond to acetylcholine, and it was sometimes necessary to set up two or three such preparations before one was found sufficiently sensitive for the assays.

Leech dorsal muscle was sometimes employed (Quastel *et al.* 1936) and found to be more sensitive, but there was not usually such good agreement between consecutive readings as could be obtained with the frog muscle. Also relaxation of the leech muscle was difficult following each contraction (see also Richter & Crossland, 1949).

Preparation of slices for assay. Preliminary experiments in which the total extractable acetylcholine of the slices was estimated, indicated the necessity to avoid too high a concentration of TCA for the denaturation and extraction of the slice since this diminished the acetylcholine contractions of frog muscle. Perchloric acid denaturation and extraction, followed by KOH precipitation of the perchlorate, left sufficient K⁺ in solution for this itself to produce a contraction of the muscle. The procedure finally accepted for the estimation of total acetylcholine in the slice was as follows. At the end of the incubation period each vessel was removed from its manometer and the electrode tissue-holder transferred into a dish of warm oxygenated phosphate-glucose saline. The slice was floated out of its holder and removed into 1 ml. of 2% (w/v) TCA in a centrifuge tube, in which it was brought into suspension with a close-fitting glass pestle, immediately by hand, and then by mechanical rotation. All manipulations from the end of incubation to the suspension of the slice were carried out as quickly as possible and occupied 30–50 sec. Following suspension the pH of the contents of each centrifuge tube was brought to 2.0–2.5, with thymol blue as external indicator. After standing 1 hr. at room temp. the tubes were spun at 0°. The supernatants were decanted off, brought to pH 4.0 (bromocresol green), and stored at –15°. Immediately before acetylcholine assay the slice extracts were brought to pH 7.0 and diluted

to a known volume. Usually the extract of a slice, 80–100 mg., was made up to 2.0 ml., and except where it was required to observe the effect of cholinesterase on the extracts, eserine sulphate was included to give a final concentration of 0.001 %. For assay a measured portion of this 2.0 ml. was diluted to a known extent, of the order 1:10, with eserinated frog-Ringer.

Preparation of media for assay. Incubation media were separated promptly from the slices and filtered into ice-cooled test tubes. They were stored at -15° . In eserinated phosphate-glucose saline, acetylcholine concentrations of 0.05, 0.07 and 0.10 $\mu\text{g.}/2$ ml. applied to frog muscle, and 0.03 and 0.05 $\mu\text{g.}/2$ ml. applied to leech muscle gave the same contractions, within the error of the method, as equal acetylcholine concentrations applied in eserinated frog-Ringer. It was possible, therefore, to assay for acetylcholine by directly applying the incubation media to the muscle. Eserinated frog-Ringer was employed for dilution as assay required. Except when incubation media were applied to the muscle it was at all times bathed in frog-Ringer.

RESULTS

Effect of electrical pulses on the free ammonia of cerebral cortex in vitro

Preliminary experiments in which guinea pig cerebral cortex slices were incubated in saline with and without glucose indicated that some of the ammonia produced in the slice can diffuse into the surrounding medium. Thus in one experiment estimations on the media of slices incubated without glucose showed 2.3 mg. ammonia-N/100 g. wet weight brain slices at 15 min. and 7.5 mg. ammonia-N/100 g. at 30 min. If, therefore, exposure to electrical pulses *in vitro* gave an increase of ammonia in cerebral cortex slices it seemed likely that there would be some accumulation in the medium. Slices of guinea pig cerebral cortex were incubated, each in 5 ml. of phosphate-glucose saline. After 45–55 min. electrical pulses were applied for 20–25 min. in some cases. In individual experiments involving slices from the same animal, the incubation period for each slice was the same whether or not pulses were applied. A series of nine such experiments gave a mean value for the ammonia in the media of control slices, 6.0 ± 0.6 (S.E.M.) mg. N/100 g. brain slices, and where pulses were applied, 5.6 ± 0.7 . The difference, 0.4 ± 0.9 , was not significant ($P > 0.6$).

The ammonia content of guinea pig cerebral cortex slices has been determined after 60 min. incubation in phosphate-glucose saline. The mean of four such determinations was 0.97 ± 0.22 mg. ammonia-N/100 g. brain slices.

Effect of electrical pulses on acetylcholine of cerebral cortex in vitro

Free acetylcholine in medium. Under certain conditions electrical activity in the brain leads to a diminution in the total acetylcholine (Richter & Crossland, 1949), probably by first inducing a break-

down of the bound acetylcholine with the liberation of the free, active, readily hydrolysable form. If this sequence of events were to occur in the cerebral cortex slice, then, with eserine present to inhibit cholinesterase and with the application of electrical impulses, one would expect an increase in the acetylcholine of the incubation medium. Preliminary experiments with guinea pig cerebral cortex slices indicated that they quickly attain a stable level of total extractable acetylcholine (Table 1). It was decided to apply electrical pulses to slices which had been incubated long enough for this stable level to be attained.

Guinea pig cerebral cortex slices were incubated for 1 hr. in the absence of eserine which was added to the media at the end of the experiment. Under these conditions very small amounts of acetylcholine, sometimes $< 0.25 \mu\text{g.}/\text{g.}$ wet wt. brain slice, were found in the incubation media. In the absence of eserine, there was no consistent significant difference between the media of slices subjected to electrical pulses and of control slices from the same cortex.

However, when eserine sulphate (1:100 000) was present to inhibit cholinesterase, a significant increase ($P < 0.01$) in the acetylcholine of the medium was observed following the application of electrical pulses (Table 2, Expt. a). Another measure of the significance of the increase is that in fourteen out of the seventeen experiments in this series the acetylcholine level of the medium was more than 40% greater when pulses had been applied to the slices. The scatter of results between experiments with different animals was great, and this has been observed by others (Feldberg, 1945b; McLennan & Elliott, 1950). However, good agreement with respect to acetylcholine metabolism has been found between the right and left halves of the same brain in the course of these experiments (e.g. Table 2, Expt. b; see also Feldberg, 1945a). An increase in acetylcholine in the incubation medium,

Table 1. *Progress of formation of total extractable acetylcholine in incubated guinea pig cerebral cortex slices*

Incubations were carried out in the absence of eserine. Two slices were taken for each incubation and matched between separate incubations with regard to weight and the order of cutting from the cortex. Frog-rectus was used for acetylcholine assay.

Incubation period (min.)	Total extractable acetylcholine ($\mu\text{g.}/\text{g.}$ wet wt.)	
	Expt. 1	Expt. 2
0	< 2.0	2.9
15	4.1	5.6
30	3.9	6.1
60	4.1	6.1
120	4.2	6.0

Table 2. *Effect of electrical pulses applied to cerebral cortex in vitro on acetylcholine in eserinizied incubation medium*

In individual experiments involving slices from the same animal, the incubation period for each slice was the same and the media of two similarly treated slices were combined for acetylcholine assay in order that at least duplicate assays might be made. For guinea pig (a), mean values for a series of seventeen experiments are recorded. Assays were conducted on frog rectus in the experiments with guinea pig and rabbit, and on leech in the experiment with rat.

Animal	Measurement	Total incubation period (min.)	Final period with electrical pulses (min.)	Acetylcholine in medium ($\mu\text{g./g. wet wt. slice}$)
Guinea pig (a)	Control	75-95	—	$2.38 \pm 0.32^*$
Guinea pig (a)	Pulses applied	75-95	15-20	$3.50 \pm 0.28^*$
Guinea pig (a)	Increase on application of pulses	—	—	$1.12 \pm 0.32^*$
Guinea pig (b)†	—	75	—	$1.02 \pm 0.03^*$
Rabbit	Control	100	—	2.45
Rabbit	Pulses applied	100	40	5.42
Rat	Control	100	—	4.8
Rat	Pulses applied	100	40	7.0

* S.E.M.

† 'Inner' and 'outer' slices of both hemispheres from one guinea pig were incubated floating freely and without applied impulses. Assays were on leech muscle and the mean value for five media is recorded.

Table 3. *Acetylcholine in eserinizied incubation medium after varying periods of application of electrical pulses to guinea pig cerebral cortex slices*

In Expt. 1 frog-rectus was employed for acetylcholine assay and leech in Expt. 2.

Expt. no.	Total incubation period (min.)	Final period of application of electrical pulses (min.)	Acetylcholine in medium ($\mu\text{g./g. wet wt. of slice}$)
1	80	—	2.25
	80	10	2.93
	80	20	3.64
2	60	—	0.91
	75	—	1.52
	90	—	1.92
	75	15	3.70
	90	30	6.00

following the application of electrical pulses has also been observed with cerebral cortex slices of rabbit and rat (Table 2).

Experiments were carried out to obtain information on the time course of the acetylcholine increase in the medium following the application of electrical pulses. After preliminary incubation some slices received pulses for 2 min. In two cases the medium was taken for assay immediately following the application of pulses. The mean for the media of control slices was 2.04 ± 0.23 (S.E.M.) $\mu\text{g. acetylcholine/g. brain slice}$ and 2.04 ± 0.23 where pulses were applied. In three experiments further incubation (10-20 min.) was allowed, following the 2 min. application of pulses, because the acetylcholine, after liberation within the slice may require time to diffuse through to the surrounding medium.

The mean for the media of control slices was 3.35 ± 0.54 (S.E.M.) $\mu\text{g. acetylcholine/g. brain slice}$ and 3.30 ± 0.45 where pulses were applied. The mean decrease of 0.05 ± 0.05 was not significant ($P > 0.3$).

Experiments in which slices were subjected to different periods of applied pulses indicated a progressive increase in the acetylcholine of the medium (Table 3).

Effect of electrical pulses on the total extractable acetylcholine of cerebral cortex slices. The increase in acetylcholine found in the surrounding medium on the application of electrical pulses to cerebral cortex slices could be associated with the loss of combined acetylcholine or an accelerated synthesis, or both. To examine this point the total extractable acetylcholine of slices was measured after incubation in the absence of eserine with and without the application of electrical pulses. In experiments where the acetylcholine content of the slices has been investigated eserine has not been added, in order that the free acetylcholine may be reduced to a minimum, and in order to approximate more closely to *in vivo* conditions. The results of a series of such experiments are given (Table 4, Expt. a) and show a significant loss ($P < 0.001$) of total acetylcholine following the application of electrical pulses to cerebral cortex slices. The loss was progressive (Table 4, Expt. b).

It was of interest to know to what extent the loss of total acetylcholine from a cerebral cortex slice, following the application of electrical pulses, is reversible. A slice has been allowed a period of undisturbed incubation (50 min.), following the application of electrical pulses (30 min.), before removal from the manometric vessel into trichloro-

Table 4. *Effect of electrical pulses applied to cerebral cortex in vitro, on total extractable acetylcholine*

Cerebral cortex slices were incubated without eserine, and frog-rectus used for acetylcholine assay. (a) In individual experiments involving slices from the same guinea pig the incubation period for each slice was the same and two similarly treated slices were combined for acetylcholine assay. Mean values for a series of seven experiments are given. (b) Experiment with rabbit.

Expt.	Total incubation period (min.)	Final period for application of pulses (min.)	Total extractable acetylcholine ($\mu\text{g./g. wet wt.}$)		
			Control	Pulses applied	Loss
(a)	125-135	30	4.91 $\pm$ 0.38*	3.49 $\pm$ 0.25*	1.42 $\pm$ 0.20*
(b)	50	15	5.5	4.8	0.7
	65	30	5.5	3.9	1.6

* S.E.M.

Table 5. *Recovery of total acetylcholine in cerebral cortex in vitro, during a period of undisturbed incubation following an initial loss on the application of electrical pulses*

Guinea pig cerebral cortex slices, all 'outer' except those marked * which were 'inner', were incubated without eserine.

Expt. no.	Incubations	Total extractable acetylcholine ($\mu\text{g./g. wet wt.}$)
1 (a)	100 min. without pulses	5.0
(b)	100 min. with pulses applied for final 30 min.	3.9
(c)	150 min. without pulses	4.7
(d)	150 min. without pulses final 50 min., but following 30 min. with applied pulses	4.7
2 (e)	95 min. without pulses	4.05
(f)	95 min. with pulses applied final 30 min.	2.57
(g)	125 min. without pulses	3.70*
(h)	125 min. without pulses for final 30 min. but following 30 min. with applied pulses	3.50*

acetic acid (Table 5, Expt. 1). A control slice was incubated for the same period without any application of electrical pulses. Another pair of slices from the same cortex was employed to obtain assurance that the mechanism for the electrically induced loss of acetylcholine was operative. In this experiment all cortex slices used were samples of the 'outer' slices cut from the cerebral hemispheres. The agreement between the values for the slices (c) and (d) indicates a recovery of total acetylcholine in (d) on the cessation of electrical pulses, following a presumed initial loss indicated by the difference between (a) and (b) (Table 5, Expt. 1). A similar experiment gave essentially the same result (Table 5, Expt. 2).

Further characterization of substance contracting frog muscle

There can be little doubt that the substance estimated by its effect on frog or leech muscle, found in the incubation media or extracted from

slices, with and without the application of electrical pulses, is a choline ester. In all cases where the test has been applied, hydrolysis by boiling for 30 sec. at pH 10.0, followed by restoration to pH 7.0, has abolished completely the ability of the media and the slice extracts to bring about a contraction of frog-rectus and leech muscle. When extracts of slices which had received electrical pulses and those which had acted as controls were prepared, containing no eserine, the contractions they induced in both frog-rectus and leech-dorsal muscle increased greatly after the eserization of the muscle. The ability of these extracts to contract both frog and leech muscle disappeared on incubation at 37° for 1 hr. in frog-Ringer in the presence of cholinesterase (0.1 ml./ml.). After incubation, appropriate dilutions were made with frog-Ringer, and eserine sulphate was added to give a final concentration 0.001% before assay. Contractions were not measurably diminished after incubation with boiled cholinesterase. (I am indebted to Dr E. C. Webb for a gift of partially purified horse-serum pseudo-cholinesterase. 1 ml. of this enzyme hydrolysed 15 μmoles of acetylcholine/min. at 17° and pH 6.8.)

It is not possible to apply the cholinesterase test to the media since eserine must be present when the production of free acetylcholine is under investigation. The choline ester has been assayed in some cases, applying the same media or slice extracts to both leech and frog muscle. The two methods gave fair agreement (Table 6). In order to distinguish between acetylcholine and other choline esters several different pharmacological tests must be applied (Chang & Gaddum, 1933). The use of the frog-rectus and leech assays alone does not enable one to distinguish clearly between acetyl, pyruvyl and butyryl choline (Chang & Gaddum, 1933). Moreover, acetyl, pyruvyl (Matthes, 1930) and butyryl choline (Stedman, Stedman & Easson, 1932) are all hydrolysed readily by serum cholinesterase. However, acetylcholine is the main substance known to occur in animal tissues which would have the properties described, and it is therefore tentatively concluded that the substance assayed in these experiments is in fact acetylcholine.

Table 6. *Choline ester of incubation media and slices, with and without the application of electrical pulses, assayed with both frog-rectus and leech muscle*

Guinea pig cerebral cortex slices were incubated with eserine for media investigations, without eserine for slice investigations. In individual experiments the media of two similarly treated slices were combined or the slices combined for extraction.

Expt.	Total incubation period (min.)	Final application of pulses (min.)	Assay	Acetylcholine in medium ($\mu\text{g./g. wet wt. brain slice}$)	
				Without pulses	With pulses
(a)	90	30	Frog	1.7	3.6
			Leech	1.1	3.8
(b)	90	30	Frog	2.0	4.1
			Leech	1.5	4.3
				Total extractable acetylcholine of slice ($\mu\text{g./g. wet wt.}$)	
				Without pulses	With pulses
(c)	95	30	Frog	4.7	3.0
			Leech	5.4	3.8
(d)	85	30	Frog	2.4	1.9
			Leech	2.4	1.4

Sensitization of frog-rectus and leech muscle to acetylcholine by brain substances. It is known that there are in brain substances which will increase the sensitivity of frog-rectus (Feldberg, 1945*b*) and leech muscle (MacIntosh, 1939) to acetylcholine. The possibility has been examined that, following the application of electrical pulses, there is a decrease of sensitizing substances in cerebral cortex slices. This would give an apparent loss of total extractable acetylcholine. Acetylcholine has been removed by enzymic hydrolysis and the sensitizing ability of the extracts then tested, after the addition of a known amount of acetylcholine, by application to eserinizied leech muscle. Extracts without eserine were prepared from incubated cerebral cortex slices, with and without the application of electrical impulses. Without pulses the total extractable acetylcholine, assayed on leech muscle, was $4.65 \mu\text{g./g. wet wt.}$, with pulses $2.80 \mu\text{g./g. wet wt.}$. Samples of the extracts were incubated with cholinesterase (as described in the previous section), and it was shown that the ability to contract leech muscle had disappeared. Known amounts of acetylcholine were added to other samples of the extracts, after incubation with cholinesterase, and the contractions of leech muscle given by these solutions were compared with those given by standards of the same acetylcholine concentration. No sensitization of the muscle was observed with either extract (Table 7, Expt. 1). A similar experiment was carried out with assays on frog muscle. Without pulses the total extractable acetylcholine was $4.40 \mu\text{g./g. wet wt.}$, following pulses $3.17 \mu\text{g./g. wet wt.}$. These extracts

after incubation with cholinesterase to some extent sensitized the frog muscle to acetylcholine but each extract did so to the same degree (Table 7, Expt. 2).

DISCUSSION

Richter & Dawson (1948) found that the whole brain of the normal rat, killed by dropping into liquid air, contained $0.28 \text{ mg. ammonia/100 g. tissue}$. When rats were killed in the same way during convulsions, induced by a few seconds of electrical stimulation, this value had increased to $0.49 \text{ mg. ammonia/100 g. tissue}$. Under the conditions of the present investigations no evidence has been obtained of any increase in ammonia in guinea pig cerebral cortex *in vitro* following the application of electrical pulses for 20–25 min. However, the mean value of $0.97 \text{ mg. ammonia-N/100 g. tissue}$ was obtained for guinea pig cerebral cortex slices incubated without the application of impulses. These figures may indicate that a different balance of reactions, concerned with the liberation and uptake of ammonia, exists in the isolated slice as compared with the whole brain *in vivo*.

Present studies indicate that when electrical pulses are applied to cerebral cortex slices, incubating under good nutrient conditions, there is an increase in the output of free acetylcholine into the surrounding medium. Under the same conditions there is a diminution in the total extractable acetylcholine of the slice. The acetylcholine extractable from a slice with trichloroacetic acid is, before extraction, in a different state from that which has

pharmacological activity. In brain slices (Mann, Tennenbaum & Quastel, 1938), and probably *in vivo* (Abdon & Hammarskjöld, 1944), it is in a non-diffusible form; with respect to pharmacological tests for acetylcholine it is inactive, and it is relatively immune to the action of cholinesterase. However, this combined acetylcholine readily decomposes to liberate the free active form, e.g. by shaking with chloroform, or by standing at pH 2.0–3.0 at room temperature (Mann *et al.* 1938). It seems that the application of electrical pulses to cerebral cortex *in vitro* is one means of inducing this decomposition.

Table 7. *Sensitization of leech and frog muscle to acetylcholine by extracts of guinea pig cerebral cortex after incubation in vitro, with and without the application of electrical pulses*

Experiment 1

Extracts were prepared from slices incubated without eserine, (a) 3.0 ml. from two slices (178 mg.) combined after 100 min. incubation without pulses, (b) 3.0 ml. from two slices (177 mg.) pooled after 100 min. incubation, with pulses applied for the final 30 min. 0.3 ml. of (a) and (b) were diluted to 3.3 ml. with frog-Ringer to include 0.001% eserine sulphate before assay. Other 0.3 ml. samples of these extracts (c) and (d) were incubated with cholinesterase, eserine sulphate was added, and acetylcholine to a final concn. of 0.03 $\mu\text{g.}/2$ ml. on dilution to 3.3 ml. with frog-Ringer. A control cholinesterase incubation (e) without added extract was diluted with the same additions. Leech muscle was used for acetylcholine assays.

Solution assayed	Acetylcholine ($\mu\text{g.}/2$ ml.)
(a)	0.05
(b)	0.03
(c)	0.03
(d)	0.03
(e)	0.03

Experiment 2

Extracts were prepared from slices incubated without eserine, (f) 3.0 ml. from slices (225 mg.) pooled after 100 min. incubation without pulses, (g) 3.0 ml. from 2 slices (219 mg.) pooled after 100 min. incubation, with pulses applied for the final 30 min. 0.5 ml. of (f) and (g) were diluted to 3.3 ml. with eserinated frog-Ringer before assay. Other 0.5 ml. samples of these extracts, (h) and (i), were incubated with cholinesterase, eserine sulphate was added, and acetylcholine to a final concn. of 0.07 $\mu\text{g.}/2$ ml. on dilution to 3.3 ml. with frog-Ringer. A control cholinesterase incubation (j) without added extract was diluted with the same additions. Frog muscle was used for acetylcholine assays.

Solution assayed	Acetylcholine ($\mu\text{g.}/2$ ml.)
(f)	0.10
(g)	0.07
(h)	0.08
(i)	0.08
(j)	0.07

These investigations do not rule out the possibility that applied pulses also bring about an accelerated synthesis of free acetylcholine *de novo*. The precise extent to which the increase in free acetylcholine of the medium is determined by the loss of combined acetylcholine from the slice can only be assessed when both free (medium) and combined acetylcholine have been assayed for the same slices, with and without the application of electrical pulses. Present results indicate that the rate of increase in free acetylcholine in the medium, 0.056 $\mu\text{g.}/\text{g. tissue}/\text{min.}$ (calculated from the mean value given in Table 2) is somewhat higher than the rate of decrease of combined acetylcholine in the slice, 0.047 $\mu\text{g.}/\text{g. tissue}/\text{min.}$ (calculated from the mean value given in Table 4, Expt. a) when pulses are applied to cerebral cortex *in vitro*. It must be noted, however, that eserine was present during incubation when the acetylcholine of the medium was under investigation, but not when the combined acetylcholine of the slice was being studied.

The *in vitro* findings may be compared with the observations by Richter & Crossland (1949) of a fall in the total extractable acetylcholine of brain *in vivo*, consequent on electrical stimulation and convulsions. If electrical activity in the brain *in vivo* is associated with a release of free active acetylcholine from the combined inactive form then, since the free acetylcholine would be immediately hydrolysed under the influence of the very powerful cholinesterase of brain, the net result of such activity would be a loss in total acetylcholine. However, Richter & Crossland (1949) found the rate at which the acetylcholine of brain decreases *in vivo* on electrical stimulation, and during convulsions, to be much greater than the rates observed in the present *in vitro* studies. They found that in rats taken after 1–3 sec. of electrical stimulation there was a loss of over 50% of the normal total acetylcholine of brain, for which they give the value 1.25 $\mu\text{g.}$ acetylcholine/g. fresh brain and on the cessation of electrical stimulation this fall was followed by resynthesis of total acetylcholine of 7 $\mu\text{g.}/\text{g.}$ fresh brain/min. During convulsions they found a slower fall, to 50% of the normal value in 40–50 sec., i.e. of the order 0.83 $\mu\text{g.}$ acetylcholine/g. brain/min. This is some 10–20 times the rates of change observed in the separated tissue. Many variations in *in vitro* conditions however remain to be studied.

SUMMARY

1. In contrast with *in vivo* findings, no evidence has been obtained of any increase in ammonia following the application of electrical pulses to cerebral cortex slices respiring with glucose as substrate.

2. Electrical pulses applied to cerebral cortex slices respiring in eserinizd phosphate-glucose saline gave a progressive increase in the incubation medium of a substance acting as free acetylcholine, able to contract frog-rectus and leech muscle. Expressed as acetylcholine, the rate of increase was of the order $0.056 \mu\text{g./g. fresh tissue/min.}$

3. In the absence of eserine, electrical pulses brought about a progressive decrease in the combined acetylcholine of cerebral cortex slices (of the order $0.047 \mu\text{g./g. fresh tissue/min.}$), reversible on the cessation of applied pulses.

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Rapid Changes in Creatine Phosphate Level in Cerebral Cortex Slices

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Previous studies have shown that the application of both electrical pulses and agents such as potassium chloride and 2:4-dinitrophenol to slices of cerebral cortex results in an increase in oxygen uptake and aerobic lactic acid production and usually to a fall in the level of creatine phosphate in the slice (McIlwain & Gore, 1951; Gore & McIlwain, 1952). The changes in creatine phosphate have been measured at the end of a relatively long experimental period and give no indication of the rate at which the level is lowered, nor the rate at which creatine phosphate is resynthesized after a particular treatment. Further, it has not been possible to determine the effects of different substrates or agents such as metabolic inhibitors, convulsants or depressant drugs upon these two processes.

This paper describes a method which enables these changes to be measured during the passage of electrical pulses through slices of cerebral cortex. The results obtained have been compared with some similar changes brought about by potassium chloride.

MATERIALS AND METHODS

The saline medium contained NaCl 128 mM, KCl 6.3 mM, CaCl_2 2.7 mM, MgSO_4 1.28 mM, glucose 10.0 mM, and aminotrihydroxymethylmethane (Tris) 25 mM. The

medium was at pH 7.4. The sample of Tris was recrystallized from water before use. The saline medium contained no detectable orthophosphate when estimated as described below. In experiments in which $1.3 \text{ mM-KH}_2\text{PO}_4$ was present in the medium, the requisite amount of KCl was omitted in order to maintain the K^+ concentration at 6.3 mM. In experiments in which the concentration of KCl was increased, the control saline used with slices was made isosmotic with an equal molarity of added NaCl.

Technique for measurement of rapid changes in phosphates in slices of cerebral cortex

Slices of cerebral cortex of guinea pigs or rats were prepared as described by McIlwain (1951a). Techniques previously used for determination of changes in phosphate levels when slices were subjected to different stimuli involved too long a manipulative period to be used in the present study. An apparatus was therefore devised which permitted rapid release and fixation of a slice in a protein precipitant, after an appropriate period of stimulation. Two types of holders are shown diagrammatically in Fig. 1. Slices weighing 100–140 mg., of the cerebral cortex of guinea pigs were incubated in 3.5 ml. of Tris-glucose saline contained in 25 ml. conical flasks under an atmosphere of O_2 for 30 min. The flasks were fitted with inlet and outlet tubes closed by Bunsen valves and were shaken during incubation. The time taken between the death of the animal and placing the flasks in the thermostat was usually about 20 min. After incubation, the flask contents were transferred to a dish containing about 4–5 ml. of warm saline and the slices