Metabolic Response *in vitro* to Electrical Stimulation of Sections of Mammalian Brain

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Almost all metabolic studies of brain tissues *in vitro* have been carried out in ignorance of the electrical state of the separated tissue and without attempting to modify it. It is therefore difficult to assess the physiological significance of changes or lack of changes observed for example in respiration as a result of added substances, for the brain *in situ* exhibits continuous electrical activity. This activity can also be observed in small portions separated from certain parts of the frog brain (Libet & Gerard, 1939; Brooks, Ransmeier & Gerard, 1949), but does not appear to have been observed in the separated mammalian cerebral cortex (see de Barenne & McCulloch, 1941; Burns, 1950). In the whole animal, the electrical activity of the brain can be increased by electrical stimulation and increased metabolic activity accompanies this. More glucose and oxygen are taken from the blood stream and more carbon dioxide and lactic acid are added to it. The increased metabolic activity could thus support the greater expenditure of energy which the increased electrical activity can be supposed to involve. Changes in brain constituents can also be observed, notably in the energy-rich phosphates which also are probably involved in meeting the electrical requirements of the tissue. This subject has recently been reviewed by McIlwain (1950a).

The present studies were begun in order to see whether a similar relationship between metabolism and electrical changes which might act as stimuli could be detected in systems suitable for metabolic studies of brain tissue. (Electrical changes which might act as stimuli are referred to in the rest of the paper as 'stimuli'.) Most of the systems which have been used in the study of peripheral nerve were not suitable for this purpose (see below). Studies from these laboratories have already shown that sections of brain tissue under suitable metabolic conditions *in vitro* maintain or resynthesize the labile phosphates involved in functioning, a prerequisite for response to stimulation. If the normal relation between stimulation and metabolism is exhibited at the level of separated portions of the central nervous system, these preparations might respond to suitable electrical changes by increased respiration. We have found this to be the case.

Choice of experimental conditions

Study of the phenomena outlined above involves: (1) preparation of tissue; (2) application of electrical stimuli; (3) measurement of any metabolic response; (4) investigation of the effects of variation of the electrical stimuli and metabolic conditions. The present paper describes part of our studies of the first three of these, being concerned largely with the means of applying impulses to the separated cerebral cortex under conditions suitable for metabolic studies. To do this, we have selected the other conditions somewhat arbitrarily on the basis of existing knowledge or experimental convenience. The metabolic responses which have been measured may to some extent depend on these arbitrary choices and may not be optimal. The basis of choice was as follows.

(1) *Tissue*. The rates at which thin sections of cerebral cortex, cut parallel to the outer surface, metabolize glucose, can be very similar to those found for the brain *in vivo* (see especially Schmidt, Kety & Pennes, 1945). Such sections can resynthesize creatine phosphate and adenosine polyphosphates *in vitro* (McIlwain, Buchel & Cheshire, 1951; McIlwain, 1950b) and can maintain cozymase (Gore, Ibbott & McIlwain, 1950) although they contain enzymes which degrade these substances. They also maintain differential ion concentrations (Stern, Eggleston, Hems & Krebs, 1949; Turner, Eggleston & Krebs, 1950). Such sections were therefore chosen for the present work. The tissues are obviously to some extent damaged, and Libet & Gerard (1939) found comparable treatment reduced the spontaneous electrical activity of isolated frog brain. Nevertheless such sections are histologically relatively intact, and retain several relevant biochemical characteristics of organized structures.

(2) *Electrical stimulation*. Data are available concerning electrical, motor and other physiological responses to alternating and other forms of stimuli, applied to the cerebral or cerebellar cortex *in vivo* (see Schaefer, 1942). Most experiments have been made with cats, dogs, or primates without, however, showing marked differences with different species. Using sine-wave alternating current, Boynton & Hines (1933) found the optimal frequency for

responses at minimum voltage to be 60–90 cyc./sec.; motor responses were then found at 2–7 V. Faradic currents suitable for peripheral nerve were less suitable for the cerebral cortex. On the basis of such experiments, mains supplies of a.c. at 50 or 60 cyc./sec. have frequently been used for stimulating the brain (see, for example, Bailey & Sweet, 1940; Clark, 1939; Hines, 1940), and were therefore adopted for the present experiments.

Response to individual pulses has been found optimal with a full wave duration of 35–100 msec. and a rising phase of 6–15 msec. according to the system responding (Wyss & Alcade, 1937). For sustained activity at least 10 pulses/sec. were necessary. Rosenblueth & Cannon (1941–2) also found this to be the case for response at moderate voltage, and Moruzzi & Magoun (1949) report optimum response at minimum voltage to be given at 300 discharges/sec., and not to be given below 50/sec. This rate does not permit the individual pulses to be of the optimal duration quoted above, and the frequency used in the present experiments represents a compromise, being at 100/sec. Detailed study of the electrical characteristics of the stimuli required for optimum metabolic response does not however form part of this paper.

(3) *Measurement of metabolic response.* The manometric method has been chosen, since response to stimulation *in vitro* includes increased oxygen uptake and increased carbon dioxide and acid formation readily measured by this method. Two courses are open in devising suitable apparatus. One is to adapt apparatus which has been used for following gaseous exchange in peripheral nerve. Here, however, the rates of metabolism are lower than those of brain, and relatively little consideration has been given to exchange of metabolites between the tissue and a nutrient solution, and to their accurate determination. We chose the other course which was to use as basis the manometric apparatus in current biochemical use. This was used by Meyerhof & Schmitt (1929) in experiments with peripheral nerve, but does not appear to have been further developed. Our first type of vessel is shown in Fig. 3 and was made by sealing two electrodes through the sides of a conical Warburg vessel, whose centre well was displaced to allow for means of conducting current to a slice.

EXPERIMENTAL

General

Salines. Phosphate-glucose-saline contained 134 mM-NaCl; 5.4 mM-KCl; 1.34 mM-KH₂PO₄, MgSO₄, and CaCl₂; 13 mM-glucose; 10.4 mM-Na₂HPO₄ brought to pH 7.4 by HCl; it was saturated with O₂. Bicarbonate-saline contained 124 mM-NaCl; 5 mM-KCl, 1.24 mM-KH₂PO₄, MgSO₄ and CaCl₂; 12 mM-glucose and 26.1 mM-NaHCO₃, and was equilibrated with 5% CO₂ in O₂.

Brain tissue. Adult guinea pigs of about 400 g. and rats (Norwegian 'hooded') of about 300 g. were used. To obtain

cortex slices as large and intact as possible, the following procedure was adopted. The animals were decapitated, the hair of the head was moistened with saline, the brain bared and the cerebrum removed undamaged with a spatula and put into a Petri dish which contained also filter papers moistened with saline. It was divided into the two hemispheres and these in turn placed, lateral cortex downwards, on a filter paper moist with the saline of the experiment, and resting on a suitable support. The lateral ventricle was entered with a small spatula, thin but not sharp. With this the cortex was unfolded into a sheet of tissue; its edges were nicked with the spatula at the anterior and posterior angles to facilitate this, and slight pressure was used. The basal ganglia still projected upwards, and were cut off by a sagittal cut in the plane of the ventricle with the spatula, leaving a sheet of cortex. The filter paper carrying the cortex was picked from its support, another moist paper put in its place, and the first with its cortex was put upside down on the second and then removed, leaving the cortex with its lateral face upwards. The cortex was inspected to ensure freedom from any fragments of bone or hair, and slices were cut with a strip of razor blading, using a recessed slide as guide. Before the blade was advanced in cutting, it was drawn backwards between the cortex and guide in order to sweep air bubbles from between these two. It was easy to obtain three successive large slices from a guinea pig hemisphere, and a fourth which was sometimes less perfect.

The slices were floated from the blade or guide to a dish of the experimental saline. If necessary they were trimmed to sizes to fit the various tissue-holding electrodes described later. This was done by pressing them against the bottom of the dish by a spatula blade (of plastic or stainless steel; about 12 mm. across and rounded). They were picked from the saline by the tared wire carrier of a torsion balance, drained on a glass surface, and the carrier and slice reweighed. With the carrier they were returned to the saline and opened by shaking the carrier and sometimes also with the help of a small camel-hair brush wet with saline. The slices were then ready for fitting to electrodes as is described below.

The dry weight of slices handled in this way was found to be 0.141 of their wet weight. This is a mean ratio derived from values in twelve experiments, some of which concerned the first slice cut ('outer') and others the subsequent slices ('inner'). No difference was found between outer and inner slices.

Manometric measurements. In general the ordinary techniques were employed, using constant-volume manometers with 300 mm. scales. Much of the apparatus was specially devised for the present work and is described below. Vessels and manometers were of Pyrex glass. The general experimental arrangement is shown in Fig. 1.

Electrodes. The platinum wire used was of 26 s.w.g. (0.457 mm. diam.) except when otherwise stated, and was flattened at the points at which it was sealed through the glass, to avoid loosening on cooling. The tungsten electrodes were of wire of the same size, and after sealing were cleaned electrolytically. Gold plating on platinum was from a solution made from gold chloride (equivalent to 0.34 g. Au) and KCN (1.9 g.) in 100 ml. of water. The electrode vessel was filled with this. Plating was at 70° with a current density of 10 ma./sq.cm. or a little lower, from a 2 V. accumulator with an ammeter in series. Both electrodes in a vessel were plated together by making them cathodes; it was not possible to plate one alone as any gold, already on the other, dissolved in the cyanide mixture in absence of an adequate applied

potential. The current flowing in plating was adjusted by the extent to which the anode, a heavy gold wire, dipped to the solution. Gold, melting some 700° below platinum, could not itself be sealed to the glass vessels.

Fig. 1. A, plan of apparatus (semi-diagrammatic); B, circuit used for stimulating by condenser pulses.

Calibration of manometric apparatus. This involved determining the volume V of each vessel with its manometer to a fixed calibration point, and also that of the tissue-holding electrodes. The vessels and manometers were usually calibrated with Hg by the normal methods. No difficulty was presented by Pt or W lead-in wires. It was necessary, however, that plating with Au should follow the calibration, as Au amalgamates extremely easily. Occasional recalibration of vessels with gilded electrodes was done with water, but it was more satisfactory to remove the Au, use Hg, and replat.

For the tissue-holding electrodes, a different method was necessary. The volumes of small pieces of metal wire and gauze, when used alone, were ignored. When mounted in plastic holders, the volume of the whole—plastic, wire and any gauze—was determined by Archimedes principle. The electrode was suspended by a fine wire (stainless steel, 48 s.w.g.; 0.0406 mm. diam., was convenient) from a balance arm and weighed in and out of water at a known temperature. It was important to remove air bubbles from the apparatus, and a small camel-hair brush was used for this.

The volume of the electrode was then subtracted from V to give the volume, V_E , of the empty vessel, used for calculating vessel constants.

Electrical stimulation. (a) Sine-wave a.c. was taken from mains supply (50 cyc./sec.) through either an ordinary transformer or a 'Berco Regavolt' model 1A, which gave continuous variation in voltage although it was used at the end of its range. The ordinary transformer was arranged with about twelve steps between 1 and 5 V., and three input ranges so that a variety of output voltages was available. These and the currents were measured with a moving-coil meter and accordingly are mean and not peak values. Findings which illustrate the electrical characters of the cells used are given below. Both transformers could deliver at least 0.5 amp. without any fall in voltage.

(b) Condenser pulses were obtained from a circuit (Fig. 1, B) containing a variable capacity in series with the tissue-holding electrodes. A vibrator alternately charged the condenser from a battery, through the electrodes, and discharged it through them. The pulses thus had no d.c. component. Their characteristics were measured with a cathode-ray oscillograph.

Design of apparatus

Choice of materials for electrodes and lead-in wires.

Conducting materials were required which did not seriously change or cause serious changes when carrying a.c. currents of up to 100 ma. at a few volts through typical metabolic solutions, nor when carrying brief impulses up to 20 V. For lead-in wires the material must be sealed through glass and cleaned with the glassware. Conditions used for stimulating peripheral nerve have in general made less exacting demands than this on an electrode material, as the experiments have not involved passing such currents through some ml. of nutrient solution. The vessels used for examining electrode materials were similar to (A), Fig. 3, with lead-in wires fused high in their walls, so that liquid media did not reach the leads themselves. To the leads were attached the materials to be examined, so that these materials were partly immersed in the liquid media.

Platinum was the least suitable of all electrode materials examined. It catalysed an appreciable oxidation of glucose and of some amino-acids in the salines described above shaken at 37° in air or oxygen. Ordinary bright platinum wire was effective in doing this, and did not change in appearance during reaction. Thus, about 1 sq.mm. of platinum as wire 0.06 mm. diameter, when conducting an alternating current of 8 ma. at 3 V. through glucose-phosphate-saline caused absorption of about $10 \mu\text{mol. O}_2/\text{hr.}$ During a similar experiment in bicarbonate-glucose-saline, evolution of gas occurred. Presumably an acid is formed by the catalytic oxidation of glucose.

Gold and silver formed suitable electrodes. No gas exchange was observed with the media and currents described above, nor when much greater areas and currents were used. The metals, however, underwent a surface change where they were in contact with the salines, giving under ordinary daylight conditions a black, closely adherent film, presumably initiated at least in the case of silver by the 'chloriding' to be expected in solutions with chloride ions. This did not prevent the passage of adequate currents. On the basis of these findings silver wire and gauze were used for many of the tissue-holding electrodes; silver rather than gold, as suitable silver gauzes were available and silver has been much used for these purposes in electrophysiology. Lead-in wires were frequently of platinum electroplated with the catalytically inert gold. Gold was chosen for this rather than silver as gold was more resistant to the acids normally used in cleaning the vessels.

Tungsten and tantalum were also found suitable as electrode metals in that no gas changes occurred with the media and with the currents described above; also they could be sealed through glass.

Stainless steel could remain inert for long periods while in the polished condition in which it was purchased. Occasionally, however, patches of rusting commenced, sometimes associated with appreciable oxygen uptake. Iron, tin and lead were unsuitable as electrodes.

Choice of materials for supporting the tissue-holding electrodes. Except in the simplest case, electrodes were supported by fixing them in a plastic material. Specimens of these, both as pieces about the size of the intended holders and as chips, were shaken for some hours in ordinary manometric apparatus in (a) phosphate-saline and oxygen, and (b) bicarbonate-glucose-saline and oxygen-carbon dioxide mixture. Very small changes were observed with Polythene (a polyethylene) or Perspex (a methylmethacrylate resin), which were therefore chosen for holders. Bakelite preparations were less satisfactory.

Fig. 2. Tissue-holding electrodes. *a*, diagrammatic drawing of gauze and slice (see text); *A*, hanging gauze electrodes; *A'*, electrodes of gauze and wire (the flattened wire (*d*) can be replaced by the helix (*A''*); *A''*, helix for connecting electrodes *A'*, *B*, *C* or *D* to lead-in wires; *B*, two electrodes of type *A* fixed to a Polythene holder; *C*, *D*, wire electrodes in Perspex holders.

Tissue-holding electrodes ('holders'). Type A. It was supposed that a suitable initial design for electrodes would be one which allowed electrical contact with both poles to an appreciable area of a tissue slice, while the tissue itself should have as great as possible a contact with a well aerated saline solution, i.e. should be entirely submerged in a mechanically agitated saline, as is normal in biochemical but not in electrophysiological experiments. The simplest arrangement then was to make electrical contact with the tissue by a metal gauze. Ag gauze is available, made from wires of 48 s.w.g., or 0.0406 mm. diameter, and 0.127 mm. apart. A scale drawing of part of a tissue slice 0.35 mm. thick, in such a gauze, is given in Fig. 2, *a*. It appeared that such a gauze would not greatly disturb diffusion to

the slice. Warburg (1923) calculated that a tissue respiring at the rate of 0.05 ml. O_2 /ml. tissue/min. would receive adequate oxygen from a solution saturated with the gas at 1 atmosphere, if it was less than 0.047 cm. thick. The rate of respiration of unstimulated cortex during our experiments has been 50–65 μ mol./g./hr., or about 0.02–0.025 ml. O_2 /ml. tissue/min. It increased during stimulation up to 0.04–0.05 ml./ml./min. We have, therefore, in common with other investigators, accepted Warburg's observations and used slices of about 0.035 cm. thick, which by the same means can be calculated to be fully oxygenated when respiring at the higher rate quoted above. Field's (1948) observations are consistent with this.

The gauze used for electrodes was cut to the shape shown in Fig. 2, *A*. Two such electrodes were used for each slice. One was taken, its flaps, *b*, were partly folded, and were submerged in a dish of saline containing a tissue slice. The gauze was held at *c*, the slice manoeuvred with a spatula (blade about 12 $\times$ 5 mm.) between the jaws of the gauze, and these gently closed on it with the spatula or with forceps. In doing this, pressure was applied only at the hinge of the flaps, so that the only pressure on the slice was that transmitted by the light gauze. The second electrode was then attached to the slice. The gauze had sufficient elasticity to maintain slight pressure on the slice for long periods after being put in place. (After many experiments the holder broke at the hinge and was renewed.) The ends *c* of the two electrodes were then brought together and picked up in forceps with the slice hanging below; excess saline was drained from the whole, and it was dropped to an experimental vessel with ends *c* uppermost. They were then hung on the lead-in wires and any saline removed from the junctions by filter paper.

Such electrodes are very flexible and light (about 20–30 mg. each) and with careful handling their attachment does not visibly harm the slice. A slice was commonly torn with electrodes of similar pattern, made from gauze of stainless steel, because of the greater strength and stiffness of this material. The slice can be removed from silver holders at the end of an experiment by opening their jaws with a spatula and floating away the slice on a dish of saline. During the shaking of a manometric experiment the electrodes and slice may swing freely, or occasionally one gauze electrode becomes attached to the side of the flask by capillary attraction. In a few instances when this has happened electrical contact with the lead-in wire has been lost. This, and the desirability of maintaining the electrodes in fixed positions in relation to each other, led to making the holders described below. After initial experiments the electrodes *A* were, however, also used in a few experiments to ensure that effects of added substances were not affected by absorption on plastic holders.

Holders type B. Modifications A^1 and A^2 (Fig. 2) were made in electrodes A with the intention of ensuring contact with the lead-in wires. The contact was to silver wire, flattened to a springy clip (A^1) or curled to a helix (A^2). In each case the silver wire was flattened at its lower end and threaded through and gripped to the gauze. These electrodes were, however, found to be too heavy and rigid for handling in the manner described above. A slice tended to be torn by relative movement between the pair. They were therefore fixed in insulating holders of different sorts, and of these the most suitable was B , Fig. 2. Such holders were made from polythene sheet, their shape being chosen to allow as free as possible a circulation of saline, and to allow the holder to be put in vessels such as A and B of Fig. 3. The parts of the wires not in contact with tissue or lead-in electrodes were insulated. In use, the holders were supported in a dish of saline with their jaws submerged, open to the extent indicated in Fig. 2, and carefully freed from air bubbles. Bubbles could completely prevent the entry of a slice. One edge of a tissue slice was then floated to one jaw, this closed as before, and the other end gripped by the other jaw. The holder was then held by one electrode wire and inserted, the other end first, into a vessel. One electrode after the other was then threaded on the lead-in wires of the vessel.

Holders type C. To allow a greater variety of electrode shapes and patterns to be made with greater precision, Perspex was used as the supporting material. Holders were made of basic patterns shown in C and D , Fig. 2; modifications of this are described below. In many of these, wire rather than gauze was used to obtain contact with the slice. This was maintained on the slices at a constant pressure by the springs shown. In one case this is a helix and in the other a safety-pin or brooch-type of fastening is used. The wires held the slice against a grooved and scored portion of the holder, which ensured that the slice was fixed and in contact as much as possible with the saline. In use, the holder was immersed in a shallow dish of the saline used in the experiment and its jaws were held open by forceps which also held the whole holder firmly in position in the dish. A tissue slice was floated into position with a spatula, perhaps using also a camel-hair brush, and the jaws closed upon it as lightly as possible, and without any lateral movement which would crush and smear the tissue. Electrodes C required one end of the tissue to be put first in one electrode, and then the other end in the other.

Manometric vessels. In all cases lead-in wires ran from inside the vessel to a tube which could be filled with mercury (Fig. 3, a). Into this dipped a wire which carried the tongue of a push-in connexion. The wire and tongue were held in position by rubber tubing which also made the joint watertight. The

tongue fitted into a socket leading to the source of current. This was often through a two-pole switch to a manifold which supplied several vessels (Fig. 1).

Vessels A (Fig. 3) are those already described. When the smaller vessels carried gauze electrodes A or B , 3–4 ml. of saline were adequate to cover the slices. Aerobic or anaerobic experiments were run with the usual gas mixtures in the vessels and absorbents in the centre well. Any additions made during the experiments were made from the side arm by tipping. The larger vessels A^1 required 7–10 ml. of saline for use with typical holders.

Fig. 3. Reaction vessels. a , arrangement of lead-in electrode suitable for tissue holding electrodes A^2 , B and C (lead-in b suitable for tissue-holding electrodes A). Vessel A : conical, of about 20 ml. with side arm, two electrodes and eccentric centre well. Suitable for electrodes A and B , with approx. 50 mg. tissue and 3–4 ml. metabolic saline. Vessel A^1 : as A , but of about 35 ml. and suitable for 100 mg. tissue and 6–10 ml. saline with electrodes C and D . Centre well again eccentric. Vessel B : cylindrical, of 10–17 ml.; with two electrodes and small side arm. For applying Warburg's indirect method for CO_2 and O_2 exchange. Used with electrodes B and 3–8 ml. of bicarbonate saline.

Vessels B were used for measuring respiration in bicarbonate-containing solutions by Warburg's (1924) improved method. They differ from Warburg's apparatus in several ways. For ease of manufacture they were made round in horizontal section, and not rectangular. Valve stoppers were fitted to small side arms from which 0.1 ml. of solution could be added. This addition was adequate for many purposes; provision of larger side arms would have made the results obtainable much less accurate. Lead-in wires were provided, by which electrodes B could be attached.

Vessels C (Fig. 4) were designed to admit a tissue-holding electrode as large as possible and to provide for gas-absorbing reagents, while keeping the total volume of the system as small as possible. Here the reaction was carried out in the large centre well and the annular space used for potassium hydroxide or

other reagents. With this design lead-in wires were provided either in the vessel or in a manometer such as that shown in Fig. 4.

Fig. 4. Reaction vessels *C*; manometer with two electrodes. Vessel *C* designed to allow the use of electrodes *C* and *D* in a relatively small bulk of saline (approx. 3 ml.) in the centre chamber; absorbing reagents placed in the annular space.

Experimental procedure

The thermostat was brought to 37° and the source of electrical impulses tested. The salines were prepared and saturated with the appropriate gas mixture. The manometers to be used were greased on their cones, connected to a gas distributor and the gas flow adjusted. The reaction vessels were fitted with mercury and with the plug-in contacts; the valve stoppers were greased, any necessary addition made to the side arms and the stoppers inserted. The animal was killed and its brain sliced. As each slice was cut, a second worker trimmed the slices to size and weighed them. The first worker fitted slices to the electrodes. Salines were pipetted into the reaction vessels and the electrodes with their slices were fitted to the lead-in wires of the vessel. Any gas-absorbing reagents were added and the vessels attached to the manometers. Gas was passed for 5 min. with occasional manual shaking, the valve stoppers of the vessels closed, and the manometers and vessels put into the thermostat. After 3 min. the

vessels and stoppers were worked firm and shaking commenced. Shaking was a reciprocating motion in a horizontal plane, operated by a crank and normally at the rate of 80 excursions of 8 cm. in each direction per min. It was interrupted to make the electrical connexions and to take readings of pressure of the manometers. The first reading was usually taken after 10 min. in the bath. This was 25–40 min. after the death of the animal.

When mains a.c. was used as the source of current, a suitable transformer setting was found to give the voltage required. The output of the transformer was then connected to the vessels. The voltage and current through each vessel was measured during the experiment. When condenser discharges were used, the charging voltage was selected and measured during the manometric experiment. The characteristics of the discharge in the particular vessels used were followed during the experiment with an oscillograph.

RESULTS

Respiration of slices in tissue-holding electrodes

Holding a tissue slice in a fixed position in a manometric flask would not be expected to affect its metabolism. In Warburg's (1923) early apparatus, slices were impaled on thin glass projections from the base of the vessels. Any damage to brain tissue, and any lack of contact with the saline in aerobic experiments, would be expected to lead to lowered respiration and increased anaerobic processes.

Table 1 contains the results of about forty experiments with the apparatus described above in each of which slices from a brain were incubated (i) free, and (ii) fixed in holders of various sorts. The average rate of respiration with fixed slices was slightly higher than that with free slices. In the case of the experiments of group 10, Table 1, the increase was statistically significant. This may possibly result from the fashion in which the slice is kept permanently extended in the holders, or from some effect of the wire itself. About half the experiments concerned outer slices of cortex, with one cut surface, and about half concerned inner slices with two cut surfaces. No difference was found between the two sets of experiments.

Effects of electric currents in the absence of tissue

Several of the experimental conditions involved passing appreciable currents through different physiological salines and metabolic mixtures. The greatest currents were from mains a.c. supplies, and the effect of these on the medium alone was accordingly examined. The supply was used at up to 3.5 V. with currents up to 100 ma./vessel, but usually not more than 20 ma. In control experiments such currents have been passed between gold, silver, and polished stainless steel electrodes in each

Table 1. *Respiration of brain-cortex slices in tissue-holding electrodes*

(Normally two equal electrodes held the slice at opposite ends; the area given is the sum of each at all their points of contact with the slice (i.e. above, as well as below). Areas given for gauze are those of the gauze, not of the wires which constituted it.)

Group no.	Tissue-holding electrodes		Medium*	$\frac{\text{Respiratory rate in holder}}{\text{Respiratory rate free}} \times 100$
	Metal	Area (sq.mm.)		
	(i) Holders of wire (<i>w</i>) or gauze, hanging freely			
1	Pt	13	<i>a</i>	106, 115†, 100
2	Pt	65	<i>a</i>	110, 94, 106, 113†, 115, 96
3	Pt	400	<i>a</i>	91
4	Pt	65	<i>b</i>	110
5	Pt	65	<i>c</i>	101
6	Ag	60	<i>a</i>	120
7	Ag	60	<i>d</i>	116, 98, 100
8	Au	30 (<i>w</i>)	<i>d</i>	94
9	Stainless steel	65	<i>d</i>	92
(ii) Holders of wire (<i>w</i>) or gauze in plastic supports				
10	Ag	80	<i>d</i>	110, 110, 103, 116, 105, 105, 89, 105, 109, 100, 104
11	Stainless steel	14 (<i>w</i>)	<i>d</i>	110, 87, 100, 100, 102
12	Stainless steel	14 (<i>w</i>)	<i>e</i>	96
13	Stainless steel	36 (<i>w</i>)	<i>d</i>	102
14	Stainless steel	36 (<i>w</i>)	<i>e</i>	105

* Media: *a*, glycylglycine, glucose, Ca⁺⁺; *b*, as *a*, without Ca⁺⁺; *c*, glycylglycine, glutamate, Ca⁺⁺; *d*, phosphate, glucose, Ca⁺⁺; *e*, phosphate, glutamate, Ca⁺⁺.

† With rat cortex; all others were with guinea pig.

experimental saline in oxygen or oxygen/5 % carbon dioxide without any progressive gas change. Cellophan and filter paper have been gripped between the electrodes in some experiments. Thus neither electrolysis, oxidation, nor formation of acidic and basic materials has been caused. Untoward effects with platinum electrodes are noted above.

Gas changes due to the heating effect of the currents have, however, been observed. A typical effect is shown in Fig. 5. Passage of the current caused a small increase in pressure, which within about 2 min. was stabilized at a slightly higher value than before. It sank to its original value on switching off the current. Presumably, therefore, the vessel is maintained by the current at a temperature slightly above that of the thermostat, but no progressive increase in temperature takes place as heat is removed by the water of the thermostat. In different experiments the increase in pressure has been found to be greater with the passage of greater currents. By applying the gas laws, the mean increases in temperature in the vessels were calculated to be of the order of 0.03–0.3° in typical experiments (see Table 2). The calculation was made by simple proportionality between pressure (1 atm. = 10,000 mm. of manometric fluid) and temperature (originally 310° K.), and solubility differences were not taken into account. The validity of this calculation can be confirmed, at least approximately, by calculation on an altogether different basis. The electrical energy expended in the vessels can be obtained from the characteristics of the currents used, and the temperature gradient needed for its dissipation calcu-

Fig. 5. Respiration of (*A*) a free, outer, guinea pig brain-cortex slice and (*B*) a similar slice, stimulated. The stimulated slice was in a holder of type *B*, Fig. 2, in a vessel of type *A*, Fig. 3. Gas phase: O₂, with NaOH paper in the centre well. Medium: glucose phosphate saline with Ca. Stimulation: 50 cyc./sec. a.c. at 2 and 3 V. as indicated. Curve *C* represents the pressure change in a vessel with medium and electrode only, calculated as though the vessel contained a slice of the same weight as the experimental slices. The current causes an increase in pressure, as shown in *C*; this causes a momentary decrease in apparent O₂ uptake, as shown in *B*.

Table 2. Heating effect of currents during manometric experiments

Vessel type	Electrodes	Potential applied (V.)	Current passing (ma.)	Pressure increase (mm. of manometric fluid)	Calculated mean temperature increase (°)
Alternating current					
A	B	3.0	8	3	0.10
A	C	3.0	23	6	0.20
		3.0	48	11	0.34
B	B	1.9	13	2	0.06
		2.9	20	3	0.09
B	B	1.5	11	1.5	0.05
		2.2	17	2	0.06
Condenser discharge					
A ¹	C	6	(100/sec., 4 μ F.)	<1	<0.03
A ¹	C	24	(100/sec., 4 μ F.)	4	0.12

lated from the thermal conductivity of glass and dimensions of the vessels. The energy put to the cells by 30 ma. at 3 V. corresponds to about 0.02 cal./sec.; that by 100 pulses/sec. from a 4 μ F. condenser at 24 V., to about 0.03 cal./sec. In glass with conductivity of 0.002 cal./sec./cm. thickness/sq.cm./°, as a vessel of 60 sq.cm. area and 1 mm. thickness, loss of 0.03 cal./sec. would require a temperature difference of about 0.03°.

The mean temperature change is thus small enough to be ignored. If the temperature change were highly localized within the vessel it could still be serious, but there is no indication that such localization occurs. The current, and the heat generated by it, would be expected to be spread through a considerable proportion of the saline medium. That this is the case was shown by altering the position of the electrodes. When they were at the surface of the liquid, the current which passed between them was approximately half that which passed when they were fully submerged. This occurred whether or not the slice was gripped in the electrodes. The slice had a much lower conductivity than the medium. The effect of temperature on the respiration of slices of cerebral cortex from the rat has been studied by Field, Fuhrman & Martin (1944) and Fig. 2 of their paper shows about 30 % increase in rate between 37.5 and 42.5°. The higher rates also are not well maintained. It appears unlikely that temperature changes would cause major artifacts in the present experiments.

The increase in pressure due to heating can also be recognized as superimposed upon progressive changes of pressure due to reactions occurring simultaneously in the manometric vessels. An example in which the progressive change is caused by respiration of a brain slice not attached to the electrodes is given in Fig. 7. The pressure change due to heating can then be obtained by extrapolation, and values so obtained are close to those of Table 2. The same heating occurs when a slice is held between

the electrodes (Figs. 5–8). It can be clearly differentiated from the other changes occurring under these conditions by its magnitude, and by the fact that it occurs promptly and is reversed promptly on switching off the current.

Fig. 6. Respiration of unstimulated (A) and stimulated (B) inner slices, both in holders of type D in vessels A¹. Stimulation at 2.5 V.; other details as Fig. 5.

Respiratory response to applying potentials to nervous tissues

Increased oxygen uptake has been found to follow the application of varying potential gradients in a variety of experimental circumstances. The increase is very easily observed in the apparatus described, but one of our main concerns has been to investigate whether or not it represents a genuine response of the tissue to the applied potentials. Experiments

have therefore been arranged in a number of ways so that alternative explanations could be examined.

(1) *Comparison with control slices.* Slices in normal Warburg vessels have been included in most experiments as a criterion of the normal behaviour of

Fig. 7. Respiration of (A) a free outer slice in a vessel type A¹, Fig. 3, also containing an electrode type C, Fig. 2; (B), a similar slice held in the electrode in such a vessel; (C) a free slice in a vessel without an electrode. Other details as Fig. 5.

Fig. 8. Respiration of slices: (A) free; (B) in holders B (Fig. 2) and vessels (A) (Fig. 3); (C) as (B) but cut half-way between the two electrodes.

medium and slice. The difference during stimulation is clear (Fig. 5). Slices in holders, but not stimulated, have been compared with those which are stimulated (Fig. 6), and again show a clear effect of stimulation.

(2) *Comparison with the same slice in absence of stimulation.* Most experiments included periods with and without stimulation; the effect of the applied current is then as shown in Figs. 5–8.

(3) *Comparison with similar vessels not containing slices.* If the increase in gas absorption is due to the current alone, a progressive increase should be seen in an otherwise empty vessel in the absence of tissue. Fig. 5, however, shows that the only phenomenon under these conditions is the transitory increase in pressure presumably (see above) caused by heating. Any chance difference between vessels and electrode arrangements was eliminated by altering the arrangements so that a slice was stimulated in the vessel previously used as a control.

(4) *Comparison with similar vessels containing electrodes and slices not attached to electrodes.* This comparison was made because the slices change the composition of the medium and might conceivably produce substances labile at the applied potential. No indication of this was found. Vessels with slices attached to electrodes showed increased oxygen uptake; others did not. Fig. 7 illustrates this.

(5) *Various other phenomena* described below can also be regarded as evidence that changes observed are a response of the tissue.

Dependence of response on slice/electrode arrangements

As is indicated by the results of paragraph (4) above, a fairly close relationship between slice and electrode is needed to obtain maximum respiratory

Fig. 9. Electrode patterns (see text). B is the same as B of Fig. 2; all others were of wire fitted to Perspex holders C or D of Fig. 2. The approximate position of a tissue slice is indicated by shading.

response with a given electrode and stimulus. To obtain this, light pressure has been exerted by the electrodes on the tissue. Various arrangements of electrodes and tissue are possible of which we have examined several illustrated in Fig. 9. Table 3 shows

Table 3. Increase in respiration caused by electrodes of different types

(Exps. 1-9 were made with guinea pig cerebral cortex in glucose phosphate saline with Ca salts, and an atmosphere of O₂ in vessels *A* (Fig. 3) or *C* (Fig. 4). Exp. 10 was in glucose bicarbonate saline with Ca salts, and CO₂/O₂, in vessels *B*. Stimulation was by mains a.c. through a transformer at the voltage quoted except in Exp. 9. This employed discharge from a 4 μF. condenser of about 1 msec. duration, at the peak voltage quoted. The first period of stimulation was normally about 40 min. and the second 120-140 min. after putting the vessels into the thermostat. Respiration is calculated as 100 × rate during stimulation/mean of rate by the same slice in periods immediately before and after stimulation. The mean value before and after stimulation was 57 mol./g. wet wt./hr. corresponding to 9 μl./mg. dry wt./hr.)

Exp. no.	Electrode type (see Fig. 9)	Slice (o, outer i, inner)	Stimulating period	Voltage (V.)	Current (ma.)	Respiration (% of control)
1	<i>E</i>	o	1st	1.6	8	109
1			2nd	2.5	19	120
2	<i>E</i>	i	1st	2	23	105
2			2nd	3	36	107
3	<i>A</i>	i	1st	1.6	7	116
3			2nd	2.5	17	141
4	<i>C</i>	o	1st	2	28	138
4			2nd	3	41	160
5		o	1st	2.5	28	174
6		i	1st	1.6	10	118
6		i	2nd	2.5	24	136
7	<i>F</i>	i	1st	2.3	16	157
7			2nd	2.3	16	147
8	<i>D</i>	i	1st	2.1	90-100	208
8			2nd	2.9	120-130	200
9	<i>D</i>	o	1st	6	See above	180
9			2nd	12	See above	200
10	<i>B</i>	i	1st	2.9	13, 17	179

that electrodes (*E*), which give a relatively small area of contact with the slice and offer a fall of potential of 2-3 V. over a small space of about 2 mm., caused little stimulation. When the current passed between two parallel wires with much more of the slice between them, as in electrodes *A* and *C*, larger responses were given. The currents passing were comparable. There was not a great difference between the responses given to arrangements *A* and *C*, although a much greater proportion of the slice is between the electrodes in *C*. Increasing the area of each electrode, as in *F*, while still leaving a large proportion of the slice some mm. from each electrode, did not greatly increase the response. Greater response was, however, obtained with the grid *D*, in which each part of the slice is within 1-2 mm. of the electrode and the potential gradient is higher.

Dependence on time and length of stimulation

Long or repeated stimulation led to falling response by the tissue. The period during which a response was obtained, however, extended for some hours. An example is quoted in Table 3 in which response to a second period of stimulation at a given level was less than to the first. Also, response to a given voltage was less when this was applied late in an experiment than when it was applied early (see

Fig. 10). Maintenance of response did not markedly differ when condenser discharges were compared with the sine-wave alternating current.

Fig. 10. Respiration of guinea pig brain slices in holders *B*, Fig. 2, at varying voltages. Glucose phosphate saline in O₂. Black points and upper line: first period of stimulation beginning after about 40 min. metabolism. Open circles and lower line: second period of stimulation beginning after about 120 min. Lines drawn through mean values.

Dependence on applied potential

As is indicated above electrical energy can be expended in a vessel containing tissue without having any effect on the metabolism of the tissue.

When the tissue was fixed in close proximity to electrodes, the metabolic response varied with the voltage applied (see, for example, Fig. 7). In the electrodes examined, a threshold voltage has been found for increase in respiration; this was of the order of 1.5 V. for sine-wave a.c. (Fig. 10). Above this the response increased rapidly and then more slowly; the maximum increase observed was of the order of 100 % (Table 3).

DISCUSSION

In judging the significance of the effects reported above, it is important to compare them with existing knowledge of the response of the brain to electrical stimulation *in vivo*. Many similarities are found. Because of this it appears reasonable to refer to the applied potentials in the present experiments as 'stimuli'. The features which can be compared are as follows.

Electrical characteristics. Full examination will be reported later. In most of the present experiments the type and frequency of the stimulus has been chosen arbitrarily. However, when that choice has been made, it is significant that the voltage needed for metabolic response *in vitro* is not far from that found necessary for autonomic responses *in vivo* by Bailey & Sweet (1940); for motor responses *in vivo* by Clark (1939), Moruzzi & Magoun (1949) and Wyss & Alcade (1937), or for the electrical response *in vitro* by Libet & Gerard (1939). The comparison cannot, however, be pressed as the electrode arrangements differed among the experiments *in vivo* and differed from our own.

Promptness of response. Curves such as those of Figs. 5-8 suggest that the metabolic response is maximal within 1 or 2 min. of the commencement of stimulation. It is thus as prompt as can be detected by the present apparatus. Motor responses *in vivo* occur within fractions of a second and increased respiration of the brain *in vivo*, measured potentiometrically, occurs within half a minute (Davis, McCulloch & Roseman, 1943; Gurdjian, Webster & Stone, 1946). The findings *in vivo* and *in vitro* therefore agree within the limits of measurements at present available. Increased metabolism in the present experiments usually ceased fairly promptly on stopping the current. In some cases an increased rate may have persisted for a few minutes after stimulation.

Fatigue. Appreciable falling in response occurs only after some hours; i.e. after passing perhaps 10⁶ impulses. The tissue *in vitro*, as *in vivo*, is thus very resistant to fatigue.

Respiration. Respiration increases on applying adequate potentials both *in vivo* and *in vitro*. The magnitude of the response observed *in vitro* is within the range of metabolic potentialities recorded

for the brain *in vivo* and *in vitro*. Thus during convulsions the rate of respiration of brain as measured by arterial/venous difference can increase to 180 % of its normal values (Schmidt *et al.* 1945). The unstimulated rates of respiration of cortex slices in our experiments are similar to those of many others. They correspond to values of about 10 μ l./mg. dry wt./hr. Such rates are, however, known to be increased by glutamate (Weil-Malherbe, 1936) or by absence of calcium salts (Dickens & Greville, 1935; Canzanelli, Rogers & Rapport, 1941). By these and other modifications in physiological salines, rates of respiration of brain cortex *in vitro* have recently been observed (Krebs, 1950) which are two to three times those of ordinary unstimulated slices. The increases which we observed on electrical stimulation are therefore within the ordinary metabolic potentialities of the tissue, and can be induced by other means. Whether these other means alter an electrical state in the slices is at present unknown.

The present results can also be compared with the effects of stimuli on other separated systems. Observations with peripheral nerve are most closely analogous, and show that increased respiration also follows electrical stimulation. A new feature of the present work lies in the demonstration of such relationships after the much greater disturbance involved in slicing a highly complex organ, and the application of neurophysiological techniques to tissue slices under optimal biochemical conditions. Here it may be noted that the respiration of tissue slices from the submaxillary gland was found by Deutsch & Raper (1938) to be increased by acetylcholine.

The means by which electrical stimulation of the brain *in vivo* leads to increased respiration is through the induction of increased electrical activity by the tissue itself. The present findings suggest that cortex slices may be capable of showing induced electrical activity and that this may represent a modification of electrical conditions pre-existing in the slice. The rate of respiration of brain-cortex slices in ordinary physiological salines is closely similar to the rate found for the normal (electrically moderately active) organ *in vivo* (Schmidt *et al.* 1945; Elliott & Henderson, 1948). It has already been noted that Libet & Gerard (1939) found small isolated fragments of certain parts of frog brain to exhibit spontaneous potential changes *in vitro*, and found such changes to increase with applied stimuli. Respiration was not then measured. de Barenne & McCulloch (1941) and Burns (1950) have found electrical responses to stimulation in portions of cerebral cortex still supplied by the bloodstream of the animal, but otherwise separated; the cortex did not show spontaneous activity.

These investigations, together with our own, emphasize how autonomous are separated portions

of brain tissue. They suggest that separated portions of cerebral cortex from experimental animals or from man may be used in a wide range of investigations in which metabolic and electrical responses to stimuli are open to simultaneous investigation.

SUMMARY

1. Methods have been devised, using modified manometric apparatus, which enable electric stimuli to be applied to thin slices of brain tissue under good metabolic conditions.

2. The application for some hours of alternating currents of up to 3 V., or of brief condenser discharges of up to 30 V. several time per sec., between electrodes 2–10 mm. apart caused in the apparatus

no changes which might be mistaken for metabolic responses.

3. When current was applied to electrodes in contact with brain-cortex slices, the respiration of the slices increased. Values up to double those of the initial respiration were observed.

4. The increased respiration could be maintained for some hours, and in a given experimental arrangement increased with increase in the voltage applied, when this was above a threshold value.

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Amino-acids and Proteins in Haemoglobin Formation

2. ISOLEUCINE

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Among essential amino-acids, the role of isoleucine in haematopoiesis is specially interesting, because, despite its absence in ox, human and horse globin (Brand & Grantham, 1946), this amino-acid has been shown to be essential for haemoglobin formation in weanling rats by Orten and collaborators (Orten, Orten & Bourque, 1945; Orten, Bourque & Orten,

1945). The present paper describes studies on the influence of isoleucine on the regeneration of haemoglobin and red cells in adult rats suffering from experimental anaemia, graded doses of isoleucine being administered to rats maintained on a diet containing horse globin as the sole source of protein and rendered anaemic by administration of