CHEMICAL EXPLORATION OF THE BRAIN

A STUDY OF CEREBRAL EXCITABILITY AND ION MOVEMENT

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PREFACE

The brain has been the subject of chemical exploration for over two centuries: throughout the rise and great development of organic chemistry and of biochemistry. Successive phases of the exploration have shown its riches in unusual or intriguing chemical compounds. The names psychosine, cerebroside, kephalin and nervone are reminders of an initial period of purely chemical isolation in which the knowledge sought was of the substances present in the brain, and little immediate connection with their functioning could be given. The names aneurin, analgesic, weckamine and tranquillizer recall a further period in which connection was sought between cerebral substance or added reagent and overt behaviour. These and other phases of exploration feature in the present book but its central theme lies between the two just adumbrated. It concerns the chemical basis of cerebral excitability, and has involved the study of membrane phenomena, of ion movements and of how metabolically-derived energy is applied in supporting these central features of neural action.

The exploration recounted has been shared with many collaborators and parts have been described in lectures in London, Oxford, Paris, New York, and Los Angeles. I am grateful to investigators in all these places for helpful discussion, and also to Dr. R. Rodnight and Dr. D. B. Gammack for their comments on the typescript.

H. McILWAIN

London, July 1962

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Chapter 1

IDENTIFYING SUBSTANCES INVOLVED IN THE LOSS AND RECOVERY OF CEREBRAL EXCITABILITY

The normal brain shows continuous electrical activity and continuous consumption of chemical substances. It can be brought to greater activity in both respects by a variety of stimuli acting in physiological or psychological fashions, and also by applied agents, either chemical or electrical. Further, the excitability of the brain, or of cerebral tissues in isolation, can be lost in a variety of ways when material supplies are inadequate. Conceivably such loss might be put to good account as an experimental technique if it could be induced under defined conditions and a search then made for substances which would restore excitability. Not all conditions under which loss occurs are likely to yield systems susceptible to restoration; but of those found susceptible some should yield knowledge of hitherto unrecognized substances concerned with cerebral functioning, or give insight into the manner of functioning of known substances.

Exploration in this fashion became more feasible in 1950, when electrical stimulation of isolated mammalian cerebral tissues was first observed[1], and this Chapter describes three successive investigations which employed tissues from the brain of man or of experimental animals. These tissues can be prepared in portions of about 100 mg which retain most of their original cell structure but which are thin enough to receive their material supplies from a surrounding solution instead of from the blood stream. When the surrounding solution contains glucose, oxygen, and inorganic salts in about the quantities present in blood plasma, the tissue respires and responds to electrical excitation by an increased utilization of glucose and of oxygen[1]. An experiment showing this property in human cerebral tissues is quoted

in Fig. 1. It has the additional interest of being the first demonstration of responsiveness to electrical excitation in isolated human cerebral tissue[2,3]. Such tissue, and also that from experimental animals, is not easily depleted by stimulation: it will continue to respond during periods of an hour or more, during which time nearly a million stimulating pulses may have been applied to it. When pulses are stopped, the tissue's respiration promptly reverts to a rate close to its previous value, but can again be increased by pulses.

Because stimulation did not itself exhaust the tissue, there was next explored the effect of stimulation in absence of substances important to the tissue. If any individual constituent of the medium of Fig. 1 was omitted and tissue incubated in the

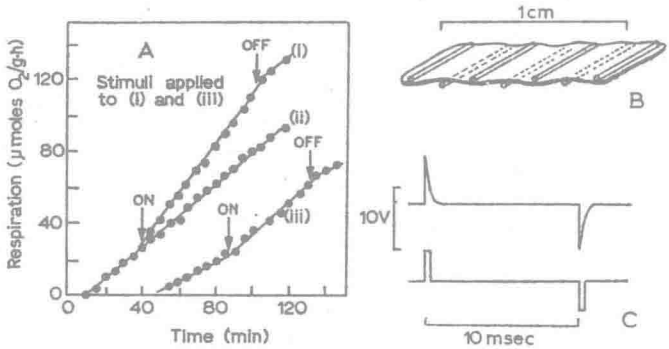

Fig. 1. *A*. Electrical stimulation of the respiration of human cerebra cortex from the temporal lobe[2], prepared as sections 0.35 mm in thickness and held between electrode grids as shown diagrammatically at *B*. In these and in several subsequent experiments, the tissues were immersed in a few ml of glucose–phosphate–saline containing (m*M*): NaCl, 134; KCl, 5.4; KH_2PO_4, $MgSO_4$ and $CaCl_2$, 1.34; glucose, 13 and a sodium phosphate buffer (pH 7.4), 10.4. This was saturated with oxygen and the whole shaken at 37°. Oxygen uptake was measured manometrically and at the points indicated electrical pulses were applied. Tissues (i) and (ii), grey matter; (iii), white. The time–voltage relationships of pulses effective in stimulation are given at *C*: above, exponential and below, rectangular. Details of electrodes, conditions of stimulation and relationships between electrical characteristics of pulses and the magnitude and speed of metabolic response, in tissues from experimental animals, are given elsewhere[1,2,5,37].

deficient medium for a period before the deficiency was made good and respiration measured, respiratory rate in the now complete medium was usually at its normal value. However, if electrical pulses were applied while the tissue was in a deficient medium, a different result was obtained. Two such situations were explored in detail, as will now be recounted.

EXCITATION IN ABSENCE OF SUBSTRATES

The first involved stimulation in absence of oxidizable substrates[4]. Glucose is the most important substance of this category, and the brain is the most susceptible of the major organs of the body to diminution in the level of blood glucose. In several animal species including man, cerebral activities rapidly fail in severe hypoglycaemia and after some minutes or an hour, glucose may not be adequate for full restoration. Anticipating some corresponding phenomena *in vitro*, cerebral tissues were examined in the fashion indicated in Fig. 2A.

Here are compared the behaviour of two groups of guinea pig cerebral tissues both initially without glucose, but to one of which were applied electrical pulses sufficient to stimulate had glucose been present. In the absence of glucose the pulses had little effect on respiration, but the tissue so treated showed slightly lower rates when, subsequently, glucose was added (second period, Fig 2A). Electrical stimulation then revealed an even greater difference between the two groups of tissue: those previously stimulated gave little or no response while the others gave a normal increase of nearly 100%.

Substances restoring excitability

Regarding the treated tissues (ii, Fig. 2) as a deficient system, attempts were made to restore their respiratory response by adding various extracts and substances of natural occurrence. These included extracts from the brain itself and from blood, and among pure substances, compounds known to be released from neural systems on stimulation, as acetylcholine. Coenzymes

and intermediary metabolites were also included, and of some thirty substances examined, the first found to increase response was *fumaric acid*. At 10–20 m*M*, response increased to 30–40%, whereas a wide variety of materials gave no increase or an increase of no more than 10%. Even the closely related succinate is in this category (Fig. 2B), as also is pyruvate. The greatest

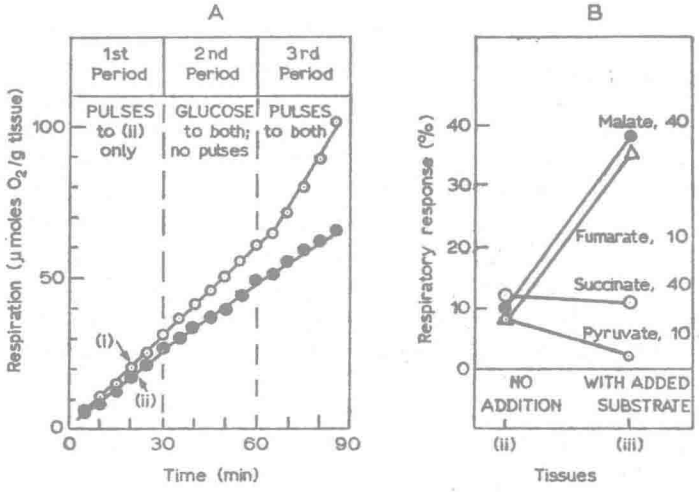

Fig. 2. Defect induced by electrical stimulation in absence of glucose[4].
A. Specimens (ii) of guinea pig cerebral cortex in the apparatus of Fig. 1, but initially in media which lacked glucose, received electrical pulses during the first period of 30 min. Specimens (i) were identical but did not receive pulses. Glucose was then added to both groups of specimens at the beginning of the second period and pulses were applied during the third: tissues (ii) gave little response.
B. Tissues (ii) were treated as tissues (ii) in *A*; tissues (iii) received with the glucose at the beginning of the third period, the additional substances named (m*M*). The respiratory response quoted is the % increase in rate induced by pulses applied during the third period.

recovery achieved was thus only partial, and depletion of the tissue as in the first period of Fig. 2A was, therefore, examined with a variety of impulse types, changing the potential and

duration of individual pulses and the time during which they were applied. In no situation was fumarate much more effective than indicated in Fig. 2; these experiments did however show that the property found in fumarate was not narrowly dependent on particular pulse types.

Of related compounds only malate acted as did fumarate[4]; it was effective when given in rather greater concentration, again together with glucose. Lactate, like pyruvate, glutamate and succinate, was ineffective. These results suggested that in the depleted tissue fumarate or malate might be performing an already well-established role of acting as an essential reactant in the tricarboxylic acid cycle. Of the substances named, fumarate and malate are closest to oxaloacetate, which is the essential reactant at the point of entry of substances being oxidized by the cycle. The substance oxidized reaches oxaloacetate as acetylcoenzyme A, which in cerebral tissues would be derived from pyruvate; but as pyruvate was ineffective in restoring the depleted tissue it presumably was available in adequate quantities from the glucose supplied.

Chemical exploration of the depleted tissue

The suggestions just made could be examined by exploring other properties of the tissue depleted by pulses in absence of glucose and the following studies were therefore carried out; the first concerned tissue glycolysis.

A proportion of the glucose metabolized by the brain *in situ* or by preparations from it *in vitro* normally leaves the brain or the tissue as lactic acid. The amount of glucose converted to lactate in the brain is greatly increased by electrical stimulation, and this property is retained by isolated tissues when these are examined under the conditions of Fig. 1 (refs. 5 and 6). Formation of lactic acid is also increased when oxygen is not available, but under ordinary aerobic conditions, increase of lactic acid with electrical pulses is one of the many metabolic properties of cerebral tissues which can serve as a measure of their response to stimulation. It was, therefore, noteworthy that the tissue

depleted by pulses in absence of glucose was still capable of glycolysis, and still afforded a large glycolytic response to further stimulation[4]. Thus, rates of lactic acid formation (μmoles/g tissue) during the third experimental period of experiments arranged as in Fig. 2A were: no pulses during this period, 12.5; with pulses, tissues not depleted, 40.5; with pulses, tissues depleted, 45.5. The defect in the depleted tissues was thus a specific one, concerning one particular process which supported the tissue's response to excitation.

The route by which respiration and glycolysis are considered to support the tissue's ability to respond to excitation involves as intermediates the energy-rich phosphates of the tissue[5,7]. These comprise several substances of which the major components are adenosine triphosphate and phosphocreatine; and of the two compounds, which also undergo rapid changes on electrical stimulation of the tissue, phosphocreatine is the most sensitive to adverse conditions. The tissues depleted by pulses in absence of glucose were, however, relatively normal in regard to phosphocreatine[4]. At the end of the second period of experiments such as those of Fig. 2A, they had resynthesized some 1.5 μmoles of phosphocreatine/g treated tissue (corresponding to 1.9 μmoles/g fresh tissue). In this respect they did not differ from the control tissues which in the first period had also been without glucose, but which had received no pulses. Fumarate in increasing respiratory response, did not increase phosphocreatine.

Appraisal

Electrical stimulation in absence of glucose has thus induced an interestingly specific defect in the tissue, but one which is more novel in relation to the means of depletion than in relation to the defect induced. For Banga, Ochoa and Peters[8,9] had shown several years earlier that if cerebral tissues were ground, suspended in salines and washed or dialysed, they required fumarate for maximal rates of respiration: observations which contributed to establishing the tricarboxylic cycle as the major route of carbohydrate oxidation in the tissue[9,10].

Presumably in both the present experiments and in those of Peters, fumarate was necessary as source of oxalacetate for pyruvate metabolism because oxalacetate had been lost by diffusion or metabolism. In the tissue subjected to grinding and dialysis, loss appears fairly certainly due to diffusion. Diffusion of such a substance may very well be altered when tissue cells undergo the polarization changes associated with stimulation, but probably not in a sense of favouring loss of an anion. Thus, in the electrically stimulated tissue, loss appears more likely to be due to metabolism of the oxalacetate. Oxalacetate is very effective as sole oxidizable substrate in cerebral tissues, giving high respiratory rates and maintenance of phosphocreatine[11]. It undergoes spontaneous as well as enzymic decarboxylation to pyruvate[12] and thus provides the two necessary substrates for the tricarboxylic acid cycle. This cycle is self-regenerating while glucose is present to provide pyruvate, but not when these substances are absent; any remaining oxalacetate could then be lost by the route indicated. It is specifically the application of pulses in absence of glucose which induced the deficiency made good by fumarate. Relationship between these mechanisms and tissue excitability is appraised after describing the investigations of the following section.

EXCITATION IN THE ABSENCE OF OXYGEN

Continuing the attempts to deplete cerebral tissues by electrical stimulation under adverse conditions, pulses were applied in absence of oxygen[13]. Activities of the brain *in situ* are extremely sensitive to lack of oxygen, consciousness being lost in less than a minute, and after a few minutes' hypoxia many of the changes become irreversible.

With isolated guinea pig cerebral cortex under anaerobic conditions, in normal media such as that of Fig. 1, the major metabolic reaction taking place is the formation of lactic acid from glucose. This, as noted above, is greatly accelerated anaerobically, a normal rate of some 15 μmoles lactate formed/g tissue/h

reaching a value of 150 μmoles/g · h in absence of oxygen (Fig. 3): indeed seeing the potency of the tissue's glycolytic systems displayed anaerobically, specific explanation of the lower aerobic rate becomes necessary, an explanation which has been given in terms of inorganic phosphate and phosphate acceptors[5,7] (see below).

Although electrical pulses increase the rate of glycolysis aerobically as in tissues (i), Fig. 3, they were not found to increase the already high rate of anaerobic glycolysis. On the contrary, they decreased it: not immediately, as was observed with the aerobic change, but gradually over a period of some 10–30 min, as is shown by tissues (ii) of Fig. 3. Within limits, the decrease was greater and more rapid with pulses of greater voltage and duration; the range of pulse-characteristics affecting anaerobic

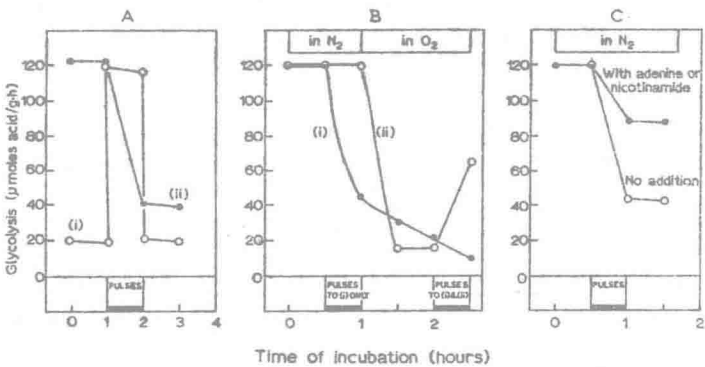

Fig. 3. Electrical pulses and the glycolysis of cerebral tissues[13]. Experiments were carried out under conditions similar to those of Fig. 1, but in media usually buffered with bicarbonate in equilibrium with O_2 or N_2 containing 5% CO_2.

A. Pulses increased aerobic glycolysis (tissues i) which reverted to its normal rate when stimulation was stopped; but anaerobic glycolysis (tissues ii) was diminished and did not revert to its previous value.

B. Pulses applied anaerobically changed the tissue so that it was unresponsive during subsequent aerobic metabolism.

C. The diminution of anaerobic glycolysis caused electrically, was antagonized by added substances.

glycolysis being similar to that affecting the aerobic changes: in respect to voltage gradient and duration of exponential pulses, and to the frequency and voltage of sine-wave currents. If pulses were stopped before they had had their maximal effect, glycolysis remained at the lower level which had then been reached.

Moreover, the tissue which had received pulses anaerobically was found to be altered in respect to its subsequent aerobic metabolism. In a normal tissue, subjected to anaerobic conditions but not to pulses and then returned to aerobic conditions, respiration and glycolysis may be a little lower than usual but both respond to stimulation. By contrast, when pulses had been applied anaerobically sufficiently to diminish anaerobic glycolysis, behaviour during a subsequent aerobic period was markedly affected (Fig. 3B). Respiration was below its normal value and it and glycolysis no longer responded to electrical pulses.

Attempted restoration or antagonism

A deficient condition had thus been induced in the tissue by electrical pulses applied in absence of oxygen, and restoration of excitability was again attempted. Restoration did not occur spontaneously after incubation for various periods of time. Nor was it induced to any appreciable extent when a variety of materials were incorporated in the incubation media during the period after pulses had been applied anaerobically. However, antagonism to the anaerobic effects of pulses was achieved.

When pulses were applied to the cerebral tissues in media which contained not only the usual salts and glucose, but also some added compounds or tissue extracts, a few of these limited the loss of glycolysis caused electrically. This is illustrated in Fig. 3C. Tissue extracts had complex effects, some themselves lowering anaerobic glycolysis, possibly through their content of glutamic acid, glutamine, or potassium salts[13]. The first defined substances observed to antagonize the diminution of glycolysis were anticonvulsive drugs[14]. Diphenylhydantoin and trimethadione at concentrations of 0.1–1 mM, which approximate to those involved in their use as anticonvulsants, limited the fall

in anaerobic glycolysis brought about by sine-wave currents of particular frequencies.

These findings, however, gave no direct explanation of the effects of tissue extracts, and accordingly a search was made among materials of natural occurrence for possible antagonists to the anaerobic effects of pulses. Fumaric and malic acids, previously found to make good, in part, the defect induced electrically in absence of glucose, were without effect in the present system. The first substance of natural occurrence which was found effective was nicotinamide, whose action is illustrated in Fig. 3C. This compound was examined in the knowledge that the nicotinamide coenzymes can be limiting factors in anaerobic glycolysis, in cerebral[15], as in other tissues. Their rôle in this respect had been exhibited in two types of system. In one of these, ground cerebral tissues had been washed or dialysed, and the ability of the tissue residue to convert glucose to lactic acid had been examined. There were required for this process magnesium salts, hexose diphosphate, adenosine triphosphate and the nicotinamide–adenine dinucleotide. Secondly, if cerebral tissues were simply ground and incubated, their ability to glycolyse failed, but the failure could be prevented by nicotinamide [16,17,18]. This was found due to a potent enzyme which degraded the dinucleotide with liberation of nicotinamide, and which was inhibited by nicotinamide.

In this knowledge, the antagonism to anaerobic effects of pulses which had been observed in nicotinamide was sought also in other substances[13]. Most of these were ineffective, but activity was found in adenine. Adenine, like nicotinamide, when present in glucose salines surrounding tissues to which pulses were applied anaerobically, minimized the fall in tissue glycolysis (Fig. 3C).

Changes in tissue constituents

Basis for the anaerobic effects of electrical pulses and for the actions of the substances which antagonize them, have received only limited further investigation as the studies concerned were