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# Actions of Electrical Stimulation and of 2:4-Dinitrophenol on the Phosphates in Sections of Mammalian Brain *in vitro*

BY H. McILWAIN AND MARION B. R. GORE

*Biochemical Laboratories, Institute of Psychiatry (British Post-Graduate Medical Federation, University of London), Maudsley Hospital, London, S.E. 5*

The expectation that sections of brain *in vitro* might respond to electrical stimulation was based on the finding (McIlwain, Buchel & Cheshire, 1951) that resynthesis of labile phosphates could be induced in such preparations. When metabolic response to stimulation was found (McIlwain, 1951 and McIlwain, Anguiano & Cheshire, 1951) our working hypothesis was that the increase in respiration and glycolysis was secondary to breakdown of labile phosphates. The labile phosphates had presumably been depleted in supporting additional activity in the slices; their breakdown products stimulate both respiration and glycolysis (for references see McIlwain, 1950). The possible nature of the additional activity is discussed below.

An immediate test of such hypotheses was to examine the concentrations of inorganic and creatine phosphates in slices of brain stimulated electrically. Very little data are available on concentrations of phosphate fractions in surviving tissue slices during *in vitro* metabolism, but the value of such data in understanding the overall metabolism of nervous tissue has been shown by McIlwain, Buchel & Cheshire (1951) and Buchel & McIlwain (1950). In particular, we were also investigating in this laboratory the effects of substances such as 2:4-dinitrophenol which disturb the phosphorylation normally associated with respiration. We have therefore, in the present paper, compared the effects of electrical stimulation and of 2:4-dinitrophenol on phosphates, respiration and glycolysis.

## EXPERIMENTAL

Guinea pig-brain cortex was used, and prepared as described by McIlwain (1951), where details of the salines are also given. The electrode vessels employed were type $A^1$ of Fig. 3 of that paper, with the tissue-holding electrodes $D$ of Fig. 2, made of silver. Six vessels were commonly used together, of which up to four were stimulated. Each vessel held a slice of 100–120 mg., and two or three of these were combined for determination of phosphates as described by McIlwain, Buchel & Cheshire (1951). In previous determinations of phosphate after *in vitro* metabolism, dependable values were obtained so long as the slices were homogenized in trichloroacetic acid within about 30 sec. of their removal from oxygenated nutrient salines. To do this in the present experiments, the following procedure was adopted.

At the end of an experiment one stimulated vessel, with its manometer, was removed from the bath while its stimulating current was still maintained, the outside of the vessel quickly wiped dry around its joint with the manometer, and the manometer removed. The current was switched off, and the tissue-holding electrodes removed from the lead-in wires

while the electrodes and slice were still in the saline of the vessel. The tissue-holding electrodes and slice were removed with forceps and put into a shallow dish of the same oxygenated saline, the electrode opened, and the slice scooped out with a wire bent as shown in Fig. 1 *D* and mounted in a handle. When the electrode was opened the slice lay on its lower grid and the wire was of such a size and shape that it could be passed under the slice, between the wires of the lower grid and lifted out with the slice. The slice was put by means of the wire into an ice-cold test-tube homogenizer with trichloroacetic acid, operated by a second worker, and immediately disintegrated in the acid. The time from switching off the stimulating current to homogenizing was 30 sec. or less. The slice from a second manometric vessel was then treated in the same way. Commonly, to obtain sufficient tissue, more than one slice was extracted with the same trichloroacetic acid, but homogenizing of each slice followed promptly its removal from the vessel. The later slices thus metabolized 1–3 min. longer than the earlier, but this was known not to affect the phosphate levels.

Slices had been weighed before the beginning of the experiment, and in several cases the gross increase in weight on transferring them to the trichloroacetic acid at the end of the experiment was also obtained. This was done by weighing the homogenizer and its contents before the addition and after homogenizing. The increase in gross weight of the slices represented medium imbibed by or adhering to the slices. This was found to represent about 60 % of their initial wet weight. The inorganic phosphate of the medium was such that this represented an increase in the phosphates of about $0.5\,\mu$mol./g. tissue. This was small in comparison with the changes in inorganic phosphate described below, and moreover was common to stimulated and unstimulated tissues.

## RESULTS

*Electrical stimulation*

Major changes in inorganic and creatine phosphates were found to follow electrical stimulation. This is shown in Tables 1 and 2. In performing these experiments it was necessary to find first whether the electrode arrangements and additional handling of

the slices affected their phosphate contents. On the basis of previous experiments the grid type of tissue-holding electrode (Fig. 2 of McIlwain, 1951) appeared most suitable as the increase in respiration produced on stimulating in this electrode was maximal. Also the tissue was not appreciably damaged by these electrodes and could be released and collected relatively easily as is described in the experimental part. Five experiments with slices in these holders in glycylglycine-glucose salines gave mean values for inorganic phosphate of 3.30 and for creatine phosphate of $1.11\,\mu$mol./g. Values for free slices (McIlwain, Buchel & Cheshire, 1951) are 3.38 and 1.12 respectively. It can therefore be concluded that the holders and the procedure of handling the slices had no appreciable effect on the phosphates.

The experiments of Table 1 employed a.c. currents which increased respiration and glycolysis by 60–100 %. The effect on the phosphates was to lower considerably the creatine phosphate, and to raise the inorganic phosphate by about the same quantity or rather more. The ratio, inorganic P/creatine P, shown previously (Buchel & McIlwain, 1950) to be a sensitive index to changes in phosphates associated with metabolic changes, thus rises several-fold. It is instructive to compare these with changes caused by rise of temperature. As previously pointed out, an increase of the order of 0.05–0.2° may be caused by the currents used in stimulation. However, to increase respiration by 50 %, an increase in temperature of over 4° is required. Exp. 4, Table 1, shows that even this causes a very much smaller change in phosphates than is caused by electrical stimulation.

Effects of the currents were shown after brief periods of stimulation. Marked changes are seen in Exp. 3, Table 1, after 2.5 min. and in Exp. 3, Table 2, after 2 min.

*Recovery from stimulation.* It was found previously that when the stimulating current was stopped, respiration fell within a few minutes. Commonly,

Table 1. *Effects of electrical stimulation and temperature change on respiration, glycolysis and phosphates*

(Experiments were in glycylglycine-glucose saline with about 20 mg. tissue/ml. All slices of Exps. 1–3, stimulated and unstimulated, were in tissue-holding electrodes. Slices from three vessels, whose respiration was measured separately, were combined for determination of phosphates by Ca-ethanol separation. Portions of their salines were combined for determination of lactic acid. Currents (sine-wave a.c.) were applied in Exps. 1–3 about 5 min. after the vessels were put at 37°.)

| Exp. no. | Temp. (°) | Voltage applied | Duration (min.) of Experiment | Duration (min.) of Stimulation | Respiration ($\mu$mol./g./hr.) | Lactic acid ($\mu$mol./g.) | Inorganic phosphate ($\mu$mol./g.) | Creatine phosphate ($\mu$mol./g.) | Ratio inorganic P / creatine P |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 37 | 0 | 40 | — | 56, 64, 54 | 41 | 4.0 | 1.18 | 3.5 |
| 1 | 37 | 2.7 | 40 | 35 | 104, 96, 98 | 61 | 5.0 | 0.27 | 18.1 |
| 2 | 37 | 0 | 30 | — | 75, 69, 69 | 43 | 3.03 | 1.10 | 2.75 |
| 2 | 37 | 2.9 . | 30 | 25 | 138, 141, 153 | 79 | 4.54 | 0.51 | 8.9 |
| 3 | 37 | 0 | 32 | — | — | — | 4.1 | 1.10 | 3.6 |
| 3 | 37 | 2.8 | 32 | 2.5 | — | — | 5.3 | 0.64 | 8.6 |
| 4 | 37 | 0 | 60 | — | 56, 56, 63 | 37 | 3.0 | 1.19 | 2.5 |
| 4 | 41 | 0 | 60 | — | 75, 75, 73 | 50 | 3.8 | 1.00 | 3.8 |

Table 2. *Recovery from effects of electrical stimulation*

(Experimental arrangements: as Table 1 except where indicated otherwise. Most of the periods of metabolism were too brief to obtain accurate measurements of the rate of respiration, but where observable, stimulation was found to lead to a marked increase of the order of that recorded in Table 1.)

| Exp. no. | Period (min.) | Voltage | Lactic acid ($\mu$mol./g.) | Inorganic phosphate ($\mu$mol./g.) | Creatine phosphate ($\mu$mol./g.) |
|---|---|---|---|---|---|
| 1a | 0–40 | 2·9 | 56 | 3·48 | 1·35 |
| 1b | 0– 5 / 5–40 | 0 / 2·9 | 125 | 5·4 | 0·57 |
| 1c | 0– 5 / 5–25 / 25–40 | 0 / 2·9 / 0 | 115 | 4·3 | 0·78 |
| 2a | 0–65 | 0 | 47 | 4·3 | 1·25 |
| 2b | 0–25 / 25–45 | 0 / 2·7 | 57·1 | 5·5 | 0·61 |
| 2c | 0–25 / 25–50 / 50–65 | 0 / 2·7 / 0 | 67·2 | 4·7 | 1·00 |
| 3a | 0–43 | 0 | — | 3·5 | 1·51 |
| 3b | 0–21 / 21–23 | 0 / 2·8 | — | 5·4 | 0·87 |
| 3c | 0–21 / 21–23 / 23–43 | 0 / 2·8 / 0 | — | 3·7 | 1·22 |

Table 3. *Concentration and action of 2:4-dinitrophenol*

(All salines contained glucose, and the dinitrophenol where indicated was present before the tissue was added. Exp. 3 employed Warburg's indirect method for gaseous exchange. Experiments were at 37° for 60–90 min., during which time respiration was linear.)

| Exp. no. | Medium | Dinitrophenol (M) | Respiration ($\mu$mol./g./hr.) | Lactic acid ($\mu$mol./g./hr.) |
|---|---|---|---|---|
| 1 | Phosphate | 0 | 60 | 22 |
| 1 | Phosphate | $9 \times 10^{-6}$ | 93 | 35 |
| 1 | Phosphate | $4 \cdot 5 \times 10^{-5}$ | 139 | 49 |
| 1 | Phosphate | $2 \cdot 3 \times 10^{-4}$ | 24 | 86 |
| 2 | Phosphate | 0 | 67 | 19 |
| 2 | Phosphate | $5 \times 10^{-5}$ | 121 | 37 |
| 3 | Bicarbonate | 0 | 62 | 42 |
| 3 | Bicarbonate | $2 \cdot 1 \times 10^{-5}$ | — | 55 |
| 3 | Bicarbonate | $5 \times 10^{-5}$ | 120 | 73 |
| 4 | Glycylglycine | 0 | 60 | 30 |
| 4 | Glycylglycine | $2 \times 10^{-5}$ | 115 | 44 |
| 4 | Glycylglycine | $5 \times 10^{-5}$ | 125 | 52 |
| 4 | Glycylglycine | $1 \times 10^{-4}$ | 95 | 73 |
| 4 | Glycylglycine | $2 \times 10^{-4}$ | 69 | 97 |

Table 4. *2:4-Dinitrophenol on phosphates*

(Glycylglycine-glucose saline was employed at 37°, with about 30 mg. tissue/ml. Phosphates in Exps. 1 and 2 were by differential stability and in Exp. 3 by Ca-ethanol separation.)

| Exp. no. | Period of metabolism (min.) | Period of contact with 2:4-dinitrophenol (min.) | Dinitrophenol (M) | Inorganic phosphate ($\mu$mol./g.) | Creatine phosphate ($\mu$mol./g.) |
|---|---|---|---|---|---|
| 1a | 75 | — | 0 | 3·8 | 1·19 |
| 1b | 75 | 75 | $10^{-5}$ | 5·0 | 0·29 |
| 2a | 75 | — | 0 | 3·65 | 1·51 |
| 2b | 75 | 75 | $10^{-4}$ | 5·30 | 0·58 |
| 3a | 45 | — | 0 | 3·0 | 1·0 |
| 3b | 45 | 0·5–2 | $5 \times 10^{-5}$ | 5·5 | 0·22 |
| 3c | 45 | 0·5–2 | $5 \times 10^{-5}$ | 4·7 | 0·50 |

rates approximately equal to those before stimulation were reached in 5 min., but sometimes a higher rate persisted. Phosphates have been found to show a partial return to normal within 15 min., which was not, however, complete in 25 min. In Exp. 1, Table 2, stimulation was commenced after only 5 min. at 37°. The creatine phosphate of freshly cut slices is low and may not, therefore, have been completely resynthesized before stimulation was applied. In Exp. 2 a period of 25 min. is allowed for the initial resynthesis and this period is known to be fully adequate. Stimulation then halved the values for phosphates, and in a 15 min. recovery period a large part of the loss has been made good. In each case inorganic phosphate was increased by stimulation and lowered in the period of normal metabolism following stimulation. Very similar changes are seen to follow a brief period of stimulation, in Exp. 3.

### 2:4-Dinitrophenol

The action of dinitrophenol on glucose metabolism by guinea pig-brain cortex was found to be similar to that recorded in other systems, and included (Table 3) large increases in respiration and aerobic glycolysis. Respiration was also inhibited by the higher concentrations of the drug. The change in phosphates was studied (Table 4) at concentrations of dinitrophenol which increased respiration and glycolysis. Large changes in phosphates were readily observed. In Exps. 1 and 2 of Table 4 the tissues treated with 2:4-dinitrophenol were in contact with it during the whole of its period of metabolism. This led to concentrations of creatine phosphate which were one-third or less of those in slices which had been in the same medium but without the drug. Inorganic phosphate rose considerably.

The time needed for these changes was found to be remarkably short. In Exp. 3, Table 4, an ample preliminary period of metabolism had been allowed for resynthesis of creatine phosphate. 2:4-Dinitrophenol to form a solution of only $5 \times 10^{-5}$ M was then added, and the slices removed and put into trichloroacetic acid at times between 0·5 and 2 min. after the addition. This short time sufficed for the drug to penetrate the slices (some 0·35 mm. thick) and greatly reduce the creatine phosphate. Inorganic phosphate again was raised.

### Joint action of 2:4-dinitrophenol and electrical stimulation

The largest changes observed to be caused in respiration by the dinitrophenol and by applied potential gradients are similar in magnitude. So also are the changes in phosphates. The loss in creatine phosphate in the two cases cannot be entirely cumulative as each may cause the loss of the greater part of the creatine phosphate. It is therefore relevant to understanding the actions of the two agents, to see whether their effects on respiration are additive.

Fig. 1 shows that the respiratory effects are not additive. Here the initial effect of the applied potential gradient was to double the rate of respiration; later the rate fell off, but it remained much
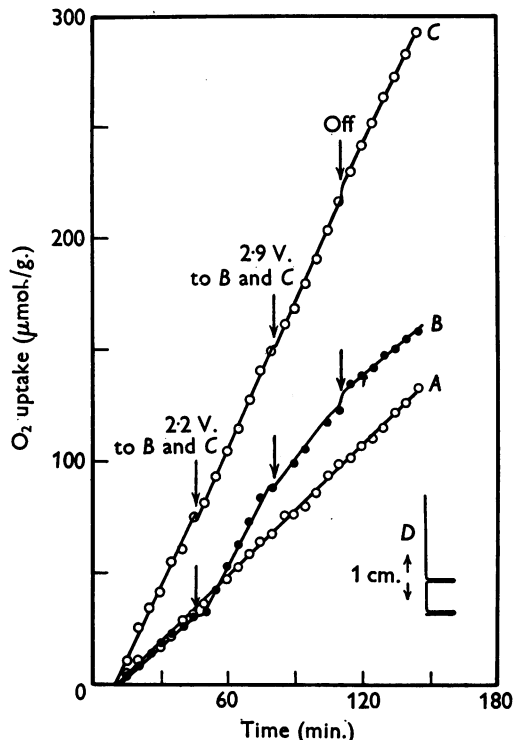


Fig. 1. $A$ to $C$, effects of dinitrophenol and of electrical stimulation on respiration of guinea pig-brain cortex slices. $D$, wire used for lifting slices from tissue-holding electrodes. The respiration was measured in glucose-phosphate saline at 37° and the slices were in tissue-holding electrodes type $D$ in vessels $A^1$ (McIlwain, 1951). Slice $A$ was unstimulated; to slices $B$ and $C$, sine-wave a.c. was applied as indicated. The medium in the case of slice $C$ contained $5 \times 10^{-5}$ M-2:4-dinitrophenol.

higher than the initial rate. 2:4-Dinitrophenol also approximately doubled respiration, and application of the same potential then had only a very small further effect. Other experiments showed that the effects on glycolysis, also, were additive to only a limited extent.

### DISCUSSION

Remarkably close similarity in biochemical changes has now been found between the effects of electrical stimulation on the cerebral cortex of intact animals, and on slices of the cortex in the newly devised experimental conditions described above. The changes measured include respiration, lactic acid formation, glucose utilization, and inorganic phosphate

formation which all increase, and creatine phosphate level which decreases. Reasons have been given (McIlwain, 1950) for considering the changes on stimulation *in vivo* to represent a coordinated series initiated by utilization of labile phosphates in supporting increased electrical activity in the cortex. Inasmuch as depletion of the labile phosphates during *in vitro* stimulation was anticipated on the basis of such conceptions, this finding can be regarded as supporting the interconnexion suggested. Moreover, the same set of changes is brought about by a very different type of agent, 2:4-dinitrophenol. The effects of this substance are known, from experiments in cell-free systems and with substances simpler than glucose as substrates, to be primarily on phosphorylation (see, for example, in the case of brain cortex, Case & McIlwain, 1951). In the present experiments the dinitrophenol can thus be regarded as leaving available for glucose catabolism a higher concentration than normal of inorganic phosphate and phosphate acceptors. At this point it could therefore influence respiration and glycolysis in the same fashion as electrical stimulation. Other interrelations between the metabolic changes observed do not appear reasonable, e.g. that increased glucose catabolism should decrease labile phosphates and increase inorganic phosphate. Examples of increase in respiration and in relevant anaerobic reactions of brain preparations caused by concentrations of phosphate and phosphate acceptors involved in the present experiments have been quoted previously (Banga, Ochoa & Peters, 1939; Long, 1945; McIlwain, Buchel & Cheshire, 1951).

The experiments with 2:4-dinitrophenol show that if one were to judge only by these metabolic effects, the actions of applied potential gradients could be due to their inhibiting phosphorylation rather than increasing the utilization of the labile phosphates formed. Experiments by different techniques are needed to decide this matter, but the similarity previously remarked (McIlwain, 1951) between the potential gradients effective *in vivo* and *in vitro* count in favour of their acting in our experiments as they do *in vivo*, i.e. by causing increased utilization of labile phosphates. Similar conclusions were arrived at with respect to peripheral nerve some time ago (Gerard, 1932).

It is also possible that any increased utilization of phosphates in our experiments might not be due to increased functional activity of the tissue. The potent adenosinetriphosphatase of brain cortex (see Gore, 1951) is noteworthy and the electrical energy applied in the present experiments might in some way facilitate its contact with its substrate without causing any increased electrical activity in the slice. However, by choosing impulses of suitable frequency and duration, much more electrical energy than that of the present experiments can be expended at a slice in a given time, without causing stimulation of respiration (unpublished observations). The balance of present evidence thus favours the suggestion that the changes observed *in vitro* bear a close relation to those observed *in vivo*. They emphasize that the increased metabolism of the brain on electrical stimulation in the whole animal need not be secondary to the vasomotor or endocrine changes which are observed to accompany it.

## SUMMARY

1. Procedures have been developed for sampling, within half a minute, sections of cerebral cortical tissue to which potential gradients have been applied during *in vitro* metabolism.

2. The potential gradients which caused increased respiration and glycolysis in sections of mammalian brain caused also a fall in their content of creatine phosphate and a rise in that of inorganic phosphate.

3. The changes in phosphates could be induced by stimulation at different times during the *in vitro* metabolism. By stopping the stimulation and maintaining good metabolic conditions for about 15 min. the inorganic and creatine phosphates were partly restored to their previous, unstimulated values.

4. The increase in respiration caused by 2:4-dinitrophenol was maximal at about a $5 \times 10^{-5}$M concentration (approx. 20 mg. tissue/ml. fluid). The maximum value was appropriately equal to the maximum respiration caused by electrical stimulation. The two effects, when each was approaching its maximum, were not additive; potential gradients normally stimulatory had negligible effects when applied to a slice metabolizing in the presence of $5 \times 10^{-5}$M-dinitrophenol.

5. 2:4-Dinitrophenol also caused a fall in the creatine phosphate and a rise in the inorganic phosphate of brain-cortex slices. It was very prompt in doing this, having a large effect within 2 min. of its addition.

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