did not take place in the absence of glucose and oxygen. In rats *in vivo*, injection of dehydroascorbic acid but not of ascorbic acid has been found to increase promptly the cerebral ascorbic acid (Patterson & Mastin, 1951). The difference between *in vivo* and *in vitro* penetration can be ascribed to the blood-brain barrier, which is not easily passed by acidic substances.

The values for cozymase proved much more stable. Its level in the separated tissue was investigated partly to ascertain whether a basis could be found for the anaerobic effect of electrical pulses in decreasing glycolysis (Table 1). This effect is inhibited by nicotinamide (McIlwain, 1956) as also is the rapid process of cozymase degradation which occurs in ground cerebral tissues. The pulses, however, had much less effect on cozymase level than on glycolysis. A partial explanation for the anaerobic effects of electrical pulses has been given by Thomas (1956).

SUMMARY

1. The total ascorbic acid of slices of rat cerebral cortex, initially 2·3 μmoles/g., fell by four-fifths on aerobic incubation in glucose saline for an hour. Most of the ascorbate lost to the tissue was found in the saline.

2. Addition of ascorbate to the saline increased the ascorbate content of the tissue, which could by this means be raised above its initial level. With external ascorbate of 30–100 μM, tissue ascorbate reached after 30–60 min. levels 8–10 times those of the fluid. The uptake of ascorbate by the tissue was greatly decreased when glucose and oxygen were absent.

3. Cozymase of guinea-pig cerebral cortex fell only about 15% during *in vitro* respiration in glucose salines, even though no cozymase or potential precursors were added; inclusion of nicotinamide did not increase tissue cozymase.

4. The cozymase level decreased anaerobically and was not increased again by various additions. It underwent little or no further change when anaerobic glycolysis was greatly decreased by electrical pulses or by potassium salts.

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The Composition of Isolated Cerebral Tissues: Creatine

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Study of isolated cerebral tissues in an electrically excited condition was based on the finding that when the tissues respired in simple oxygenated salines, their creatine phosphate and adenosine triphosphate were in part resynthesized (McIlwain, 1951, 1955). The levels reached remained, however, only about 50% of those of the tissue *in vivo*. Explanation of this is now being sought as outlined in the previous paper (McIlwain, Thomas & Bell, 1956). Of necessary precursors, creatine is known to be present at considerable level, free, in the brain *in vivo* (Hunter, 1928). Maintenance of its level is now reported in the separated tissue under different conditions of metabolism and of electrically induced activity.

EXPERIMENTAL

Salines and preparation of tissue. Glucose–phosphate and glucose–bicarbonate salines were made up as described by Rodnight & McIlwain (1954). *In anaerobic experiments*, unless otherwise stated, incubation was in bicarbonate–saline in an atmosphere of $N_2 + CO_2$ (95:5) with a stick of yellow phosphorus in the centre well of the manometric vessel. In some experiments when phosphate was to be determined the phosphate buffer was replaced by amino-trishydroxymethylmethane (tris), at a final concentration of 0·025 M, and when KH_2PO_4 was omitted from the medium the potassium concentration was maintained by increasing the amount of KCl.

Guinea-pig cerebral cortex slices of about 40 and 100 mg. moist weight were cut. For electrical treatment of tissue, slices of 40 mg. were floated on to the silver-grid holders (type H) before insertion into specially designed vessels (Ayres & McIlwain, 1953). Experiments were run with six slices from each brain, gas exchanges being followed manometrically.

Estimation of inorganic phosphate and creatine phosphate. The method was essentially the same as that of Heald (1954), based on Long's (1943) modification of the method of Berenblum & Chain (1938). For initial extraction of tissue weighing about 100 mg., 3 ml. of 10% (w/v) trichloroacetic acid at −5° was used. With 40 mg. slices it was necessary to combine two such samples.

Determination of free creatine of tissue. The method used was similar to that of Eggleton, Elsden & Gough (1943). Ennor & Stocken (1948) suggested the use of *p*-chloromercuribenzoate to eliminate interference by other materials present in tissue extracts, and this modification was adopted.

On completion of metabolic experiments, slices weighing 100 mg. were removed from vessels, drained rapidly against a watch glass to remove adhering fluid, and extracted with 3 ml. of 10% trichloroacetic acid at −5°. After homogenization and centrifuging at −5° the supernatant was decanted into a wide, stoppered tube. The residue was re-extracted with 1·5 ml. of 5% trichloroacetic acid and washings were added to supernatant. The solution was made alkaline with 7·5 N-NaOH, and 1 ml. of 0·05 M *p*-chloromercuribenzoate, 2 ml. of the alkaline α-naphthol reagent of Eggleton *et al.* (1943) and 1 ml. of 1% diacetyl were added. After making up the volume to 10 ml. with water, the colour was allowed to develop for 0·5 hr. in the dark, and was then read on a Beckman spectrophotometer at 520 mμ. Standards were run with a sample of creatine (British Drug Houses Ltd.) which had been recrystallized as the monohydrate from hot water. Controls were run with reagents alone.

For slices weighing 40 mg. extraction was with 3 ml. of trichloroacetic acid, the final volume for estimation being 7 ml. Standards were also run under these conditions.

RESULTS

Maintenance of creatine phosphate under different metabolic conditions

Table 1 gives values for the creatine phosphate and inorganic phosphate of slices of guinea-pig cerebral cortex in experiments with glucose–tris and glucose–bicarbonate salines. Under aerobic conditions the

Table 1. *Resynthesis of creatine phosphate under different metabolic conditions*

Cerebral cortex slices were in 3·5 ml. of saline in manometric vessels at 37·5°. In tris–saline the anaerobic gas phase was N_2 with phosphorus in the centre well. For aerobic recovery phosphorus was removed, replaced by NaOH and paper, and the slices were gassed with O_2 for 7 min. and reincubated. In bicarbonate, the anaerobic gas phase was $N_2 + CO_2$ (95:5) and phosphorus was put in the centre well. For recovery the centre well was empty and the gas phase was $O_2 + CO_2$ (95:5). Aerobic controls were run in both media. Alternating condenser pulses at 100/sec., peak voltage 10 v, time impulse 0·4 msec. were applied for the complete anaerobic period. Inorganic phosphate (IP) and creatine phosphate (CP) are expressed in μmoles/g. of wet tissue, followed by standard deviations with the number of observations in parentheses.

Saline	Pulses applied during anaerobic period (30 min.)	Phosphates (μmoles/g. of wet tissue)						Respiration (μmoles of O_2/g. of wet tissue/hr.)	
		Anaerobic period (30 min.)		Anaerobic (30 min.) followed by aerobic recovery period (40 min.)		Aerobic control (90 min.)		Aerobic recovery after anaerobic period (40 min.)	Aerobic control (90 min.)
		CP	IP	CP	IP	CP	IP		
Tris with KH_2PO_4	None	0·1 (3)	4·7 (3)	0·1 ± 0·02 (5)	4·7 ± 0·3 (5)	0·95 (3)	3·0 (3)	27 ± 6 (7)	56 ± 2 (12)
Tris without KH_2PO_4	None	0·12 ± 0·2 (5)	2·5 ± 0·2 (5)	0·2 (2)	2·4 (2)	1·2 ± 0·2 (11)	1·8 ± 0·3 (11)	—	—
Bicarbonate	None	0·25 ± 0·1 (7)	4·5 ± 0·2 (7)	0·6 ± 0·1 (6)	3·8 ± 0·2 (6)	1·3 ± 0·2 (6)	2·8 ± 0·2 (6)	50 ± 5 (11)*	64 ± 4 (6)
KH_2PO_4	+	0·12 (3)	4·7 (3)	0·35 (3)	4·3 (3)	—	—	—	—

* Oxygen uptake was measured by replacing bicarbonate with phosphate saline during aerobic recovery phase.

Table 2. *Free creatine concentrations of cortex slices when maintained under varying conditions of metabolism*

Slices were incubated in 3·5 ml. of saline in manometric vessels at 37·5° for 80 min. Alternating condenser pulses at 100/sec., peak voltage 10v, time impulse 0·4 msec., were applied for the latter 40 min. period of incubation. Mean values are given ± standard error with number of observations in parentheses.

Gas phase	Additions to saline medium	Electrical pulses	Creatine (μmoles/g. wet wt. of tissue)
None	(Fixed immediately after cutting)	None	10·0±0·6 (12)
O_2	Phosphate	None	1·87±0·2 (18)
O_2	Phosphate	+	1·6 (3)
O_2	Phosphate + mM glutamate	None	1·97 (3)
O_2	Phosphate + mM mixture of glycine, sarcosine, methionine and arginine	None	1·83 (3)
$O_2 + CO_2$ (95:5)	Bicarbonate	None	1·62±0·16 (5)
$O_2 + CO_2$ (95:5)	Guinea-pig serum	None	1·60±0·24 (6)
$N_2 + CO_2$ (95:5)	Bicarbonate	None	1·60±0·20 (16)
$N_2 + CO_2$ (95:5)	Bicarbonate	+	1·40±0·20 (10)

concentration of creatine phosphate is about 1·2 μmoles/g. of wet tissue during incubation for 90 min. This agrees with the previous findings of McIlwain, Buchel & Cheshire (1951), McIlwain & Gore (1951) and Heald (1954). On the other hand, an anaerobic incubation of 30 min. reduced this value to 0·2 μmole/g. Electrical pulses caused inhibition of anaerobic glycolysis of slices (McIlwain, 1956), and when applied during anaerobic experiments decreased the creatine phosphate still further to 0·12 μmole/g. of wet tissue.

Attempts were made to resynthesize the creatine phosphate of slices after anaerobiosis by reincubating aerobically for 40 min. With tris buffer there was no resynthesis, but with bicarbonate a level of 0·6 μmole/g. of wet tissue was obtained, which is about one-half the normal *in vitro* value quoted above. Tissue subjected to electrical pulses during the anaerobic period gave only 0·35 μmole/g. of wet tissue after the aerobic recovery phase.

In experiments run in tris buffer it was possible to measure the uptake of oxygen, and anaerobically depleted tissue in a subsequent period respired at a rate half that of the normal (Table 1). If the bicarbonate saline used in the anaerobic period was replaced by phosphate saline for aerobic recovery the respiration was again lower than normal tissue, but at a rate higher than in tris buffer. Electrical pulses applied anaerobically had no effect on the subsequent aerobic respiration.

Maintenance of tissue creatine

Since creatine is a precursor of creatine phosphate, and is known to occur in considerable amounts in the free form in the intact brain, it was decided to investigate the maintenance of creatine in cortex slices and its possible relationship to the resynthesis of creatine phosphate.

Table 2 summarizes the values for the concentration of creatine in cerebral slices on incubating under different metabolic conditions. When slices were fixed immediately in trichloroacetic acid levels

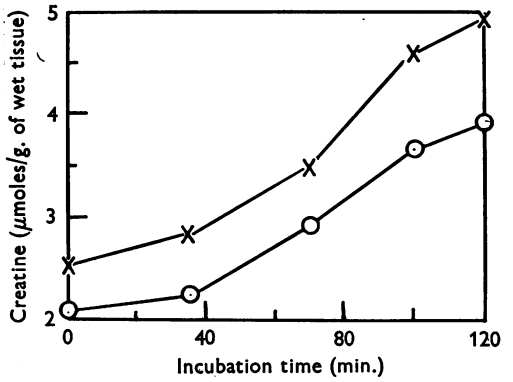

Fig. 1. Effect of time of incubation at 37·5° on the incorporation of creatine by slices from oxygenated glucose–salines buffered with phosphate (×) and bicarbonate (○), both supplemented with creatine to a concentration of 1 mM.

of 10 μmoles/g. of wet tissue were obtained. The preparation of slices involved leaving them for a period in saline before putting them into manometric vessels at 37·5°, and during this time creatine was quickly lost to the saline, leaving about 2·8 μmoles/g. of wet tissue. This was decreased to 1·8 during a subsequent metabolic incubation for 80 min., whether the suspending fluid was glucose–phosphate or bicarbonate saline or fresh guinea-pig serum. Electrical pulses applied under aerobic or anaerobic conditions had little effect on tissue creatine. Addition of mM glutamic acid or a mixture of glycine, sarcosine, methionine and arginine, which might serve as creatine precursors, also failed to restore creatine to the tissue.

When cerebral slices were incubated in oxygenated salines buffered with bicarbonate or phosphate, to which creatine had been added at 1 mM concentration [a level very much higher than that of plasma creatine (0·1 mM for human; Allinson, 1945)] there was a gradual uptake of creatine by the

Table 3. *Levels of free creatine in cortex slices incubated in salines containing added creatine*

Slices were in 3·5 ml. of saline containing mM creatine with or without glucose, buffered with phosphate or bicarbonate in vessels for 110 min. at 37·5°. For electrical pulses see Table 1. For stimulation by K^+, M/35-KCl was added to saline. Standard deviations are given, followed by number of results in parentheses.

Gas phase	Saline ± glucose buffered with phosphate or bicarbonate	Stimulation (electrical or by adding excess of K^+)	Creatine (μmoles/g. wet wt. of tissue)
O_2	Glucose, phosphate	None	4·9 (3)
O_2	No glucose, phosphate	None	2·8 (3)
N_2	Glucose, phosphate	None	2·9 (3)
$O_2 + CO_2$ (95:5)	Glucose, bicarbonate	None	3·9 (4)
$N_2 + CO_2$ (95:5)	No glucose, bicarbonate	None	2·3 (3)
$N_2 + CO_2$ (95:5)	Glucose, bicarbonate	None	2·6 ± 0·3 (11)
$N_2 + CO_2$ (95:5)	Glucose, bicarbonate	Electrical	1·8 ± 0·2 (10)
$N_2 + CO_2$ (95:5)	Glucose, bicarbonate	K^+	2·2 ± 0·3 (9)
$N_2 + CO_2$ (95:5)	Glucose, bicarbonate	Electrical	2·8 ± 0·2 (6)
$N_2 + CO_2$ (95:5) followed by $O_2 + CO_2$ (95:5)	—	None	2·2 ± 0·2 (6)

* Slices subjected to anaerobic incubation for 50 min. and aerobic recovery for 50 min. (for details see Table 1).

Table 4. *Effect of mM creatine in saline on creatine phosphate resynthesis*

Manometric vessels contained slices suspended in 3·5 ml. of bicarbonate saline with or without mM creatine. The gas phase was $O_2 + CO_2$ (95:5), the temperature 37·5° and the incubation period 2 hr. Standard deviations are given, followed by number of observations in parentheses.

Saline ± creatine	Inorganic phosphate (μmoles/g. of wet tissue)	Creatine phosphate (μmoles/g. of wet tissue)
No creatine	2·6 ± 0·4 (9)	1·5 ± 0·2 (9)
With creatine	2·4 ± 0·2 (9)	2·2 ± 0·4 (9)

slices until after incubation for 2 hr. levels of 5·0 and 4·0 μmoles/g. of wet tissue were attained respectively (Fig. 1). The creatine present in slices at the commencement of incubation was one-fifth of that *in vivo*, the loss having occurred while the tissue was standing in saline before being placed in vessels at 37·5°. This loss was greater in bicarbonate than in phosphate medium.

The uptake of creatine by slices from media containing mM creatine was influenced by the metabolic conditions used (Table 3). The effect depended on the presence of glucose and oxygen, and in salines lacking in these constituents the creatine uptake was markedly lower. Thus the creatine level of 5 μmoles/g. of wet tissue, attained by slices suspended in oxygenated glucose–phosphate saline, was reduced to 2·8 in the presence of oxygen with no glucose and to 2·9 with no oxygen but with glucose present. Similarly, in bicarbonate saline maximum assimilation of 4 μmoles of creatine/g. of wet tissue did not occur if either glucose or oxygen was absent. In oxygenated bicarbonate with no glucose the value was 2·3, and in bicarbonate provided with glucose but no oxygen, 2·6. The level was reduced still further when slices in bicarbonate were subjected to the passage of electrical pulses (1·8) or to increased potassium concentration (2·2 μmoles/g.) in the medium. An anaerobic period followed by an aerobic recovery phase produced hardly any change in the tissue creatine, but there was a slight increase in slices which had received electrical pulses anaerobically.

Cortex slices incubated in glucose–bicarbonate saline containing mM creatine not only incorporated creatine from the medium but gave higher levels of creatine phosphate. The normal *in vitro* resynthesis of 1·5 was increased to 2·2 μmoles/g. of wet tissue when the amount of free creatine available to the slices was increased (Table 4).

DISCUSSION

The preparation of tissue slices, while causing minimum cell damage, involves a partial leakage of constituents to the medium. Attempts have been made to restore some of these constituents to cortex slices, as shown by the work of Stern, Eggleston, Hems & Krebs (1949) and Krebs, Eggleston & Terner (1951) on potassium salts and glutamate, LeBaron (1955) on glycogen and McIlwain & Tresize (1956) on glycogen, glucose and lactate. In the present work it has been shown that the free creatine of cerebral slices is quickly lost when they are immersed in saline, and it is only by providing an external source of creatine very much in excess of the concentration in plasma that a partial restoration can be achieved. An analogous situation has been observed with ascorbic acid by McIlwain *et al.* (1956). Uptake by slices of creatine and ascorbate depend on the presence of oxygen and glucose, probably indicating dependence on a supply of energy.

Under *in vitro* conditions brain slices suspended in simple oxygenated saline cannot resynthesize energy-rich compounds to the fullest possible extent. This deficiency may be due to the low level of precursors available in the slice, as shown by a raising of the creatine phosphate concentration when the medium is supplemented with creatine. The restoration of cerebral constituents is markedly affected by the metabolic conditions adopted and electrical activity induced in tissue. Under anaerobic conditions of incubation there is no uptake of K^+ ions, glutamate, ascorbate or creatine, and creatine phosphate is at a low level. Cerebral slices subjected to an initial anaerobic period respired at rates below normal, and did not assimilate creatine; the extent of resynthesis of creatine phosphate was small. It is to be concluded that such treatment depletes the tissue, a depletion that is aided by the passage of electrical pulses, as shown by the decreased free creatine and creatine phosphate. McIlwain (1956) noted the inhibition of anaerobic glycolysis by electrically-treated cerebral slices, and the above change in composition appears to be part of the same phenomenon.

SUMMARY

1. The level of free creatine in fresh guinea-pig cerebral cortex was 10 μmoles/g. of wet tissue.

2. Incubation of cerebral slices in salines lowered the free creatine concentration to below 2 μmoles/g. of wet tissue.

3. Depleted cerebral slices maintained in oxygenated saline containing mM creatine for 2 hr. gave creatine levels of 5 μmoles/g. of wet tissue. The uptake of creatine from the medium depended on the presence of oxygen and glucose.

4. The addition of creatine to the medium increased the normal *in vitro* resynthesis of creatine phosphate of cerebral cortex from 1·5 to 2·2 μmoles/g. of wet tissue.

5. An initial anaerobic incubation of brain slices depleted the tissue, as shown by a reduction in respiration from 64 to 50 μmoles of oxygen/g. of tissue/hr. and resynthesis of only 0·6 μmole of creatine phosphate/g. of wet tissue during a subsequent aerobic period.

6. Cortex slices to which electrical pulses had been applied during the anaerobic period respired at a rate similar to untreated tissue but gave a lower resynthesis of creatine phosphate (0·35 μmole/g. of wet tissue). This electrical treatment also decreased tissue creatine when creatine was added to the medium.

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