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The Composition of Isolated Cerebral Tissues: Ascorbic Acid and Cozymase

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Isolated cerebral tissues in simple, oxygenated glucose salines reproduce many characteristics of the tissue *in situ*. In particular, energy-rich compounds are in part resynthesized, and this discovery encouraged search for *in vitro* counterparts to excitation. Metabolic responses to electrical stimulation were indeed found (McIlwain, 1951) but were not transmitted to the same extent as in the tissue *in vivo* (McIlwain, Gerard & Ochs, 1952; McIlwain, unpublished work). While this lack may be the result of factors other than chemical ones, it appeared pertinent to examine in more detail the composition of the isolated tissue and to attempt to make good any deficiencies found in it. Previous relevant work includes that of Stern, Eggleston, Hems & Krebs (1949) and Krebs, Eggleston & Turner (1951) on glutamate and K^+ ions; of LeBaron (1955) and McIlwain & Tresize (1956) on glycogen, lactate and glucose, and of Thomas & McIlwain (1956) on Cl^- ions. In the present paper the ascorbic acid and cozymase of rat and guinea-pig cerebral cortex are studied from this point of view.

Ascorbic acid appeared of special interest, for it is at high level in the brain. Concentrations in the hypophysis almost equal those of the adrenal, and in the cerebrospinal fluid of mammals are about twice those of blood plasma (see Lloyd & Sinclair, 1953). Dehydroascorbic acid on injection markedly affects the central nervous system (Patterson & Mastin, 1951). Cozymase has already been studied in separated cerebral tissues in this laboratory (Gore, Ibbott & McIlwain, 1950) and present observations were made to see whether its level changed with

induced changes in tissue metabolism. With both ascorbic acid and cozymase, the present observations were limited to the total quantity of substance without differentiating between oxidized and reduced forms.

EXPERIMENTAL

Tissues. The tissues were from adult rats and guinea pigs. Albino rats of about 300 g. were used, which had been maintained on rat cubes, diet no. 41, obtained from the Associated London Flour Millers. Guinea pigs weighing 400–500 g. had been fed on diet no. 18 from the same source. Animals were stunned by a blow on the neck and were bled. The brain was removed and opened, and large slices 0.35 mm. thick were prepared quickly (see McIlwain, 1951). These were weighed after draining from saline, some fitted to electrodes, and placed in the saline of the experimental vessel. Salines were those of Rodnight & McIlwain (1954), quick-transfer electrodes and procedures those of Heald & McIlwain (1956), and the grid electrodes, type H of Ayres & McIlwain (1953), wound with enamelled silver wire. This last paper also gives details of electrical pulses, manometric vessels and procedures. Incubation was at 37.5°, in manometric vessels (except when stated otherwise), in 3.5 ml. of saline. This was usually oxygenated; in anaerobic experiments N_2 or $N_2 + CO_2$ was used with yellow phosphorus in a centre well.

Estimations. Lactic acid was determined according to Barker & Summerson (1941). For ascorbic acid estimation the tissue and a sample of saline were placed in 4% (w/v) trichloroacetic acid and the method of Roe & Kuether (1943) was applied. Total cozymase was determined with apozymase (Axelrod & Elvehjem, 1939) after extraction with hot buffered nicotinamide (Gore *et al.* 1950). A specimen of cozymase from Sigma Chemical Co. was used as substrate and as standard in the apozymase assay. Spectrophotometric determination showed it to be of 75% purity.

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RESULTS

Ascorbic acid

Rat cerebral cortex sampled promptly had 2.28 ± 0.08 (S.D.; 6 animals) μmoles of total ascorbic acid/g. fresh wt., which is within the rather large range of values previously reported for rat brain (see Lloyd & Sinclair, 1953). On incubating such tissue, sliced, in oxygenated glucose-phosphate saline, its ascorbate fell rapidly so that after 10 min. only a third remained; this later fell to a fifth (Fig. 1). A large part of the ascorbate lost was found in the saline which, initially without ascorbate, increased to 30–50 μM , this representing a gain of some 140 μmoles from the 40–50 mg. of tissue, or the greater part of its initial content. Nevertheless, it will be noted that the distribution of ascorbate in the system is in favour of the tissue, for its volume is only about one-sixtieth of that of the fluid.

As the normal levels of ascorbate in blood plasma and in cerebrospinal fluid are 30–100 μM , effects of increased ascorbate in the saline were examined. The additions promptly caused increases in tissue ascorbate (Fig. 1). Uptake increased with time during the 30–60 min. of observation; with mM ascorbate the level in the tissue rose above its initial value. This gain, however, required more than the presence of ascorbate in a balanced salt mixture, for

Fig. 1. Ascorbic acid content of rat cerebral cortex during incubation in phosphate salines, some of which contained ascorbic acid added initially at the concentrations quoted beside each curve. Except in the instance noted, the saline contained glucose and was exposed to O_2 .

if the tissue was incubated without glucose and O_2 it did not recover its original level of ascorbate even when the relatively high level of 1 mM was provided in the surrounding fluid.

The activity of the tissue in assimilating ascorbic acid is illustrated in Fig. 2 by the ratio of ascorbate in the tissue to that in the fluid. The upper two curves concern levels of external ascorbate which reach final values corresponding to those normal to blood plasma or cerebrospinal fluid. After 10 min. exposure to such fluids, equilibrium had not been reached, but between 30 and 60 min. relatively stable values were observed with external ascorbate concentrations of 30 or 100 μM . The concentration ratio of 8–9 in favour of the tissue is rather lower than the values of 10–20 which normally appear to exist between blood plasma or cerebrospinal fluid, and the brain, *in vivo*. *In vitro* ratios were markedly lower with an external ascorbate of 1 mM, though the actual levels in the tissue were higher.

Cozymase

The cozymase level of guinea-pig cerebral hemispheres was initially 240 μM -moles/g. (S.D. 15; seven animals). After cutting slices and placing them in saline for 5 min., values of 170 μM -moles/g. (S.D. 19; 20 slices, seven animals) were found. The slices under these conditions imbibe saline and increase in weight by 20–25 % (Rodnight & McIlwain, 1954); thus the true loss in cozymase is not more than

Fig. 2. Tissue ascorbate in the experiments of Fig. 1, expressed as the ratio tissue content ($\mu\text{moles/g.}$)/fluid concentration (mM). Tissues were in oxygenated glucose saline for the periods shown. The initial ascorbate concentration in the saline is quoted beside each curve in the figure.

10–15 %. Thereafter, incubation in glucose saline aerobically had little effect on cozymase level (Fig. 3). Moreover, several procedures which altered the level of metabolic activity in the tissue had little or no effect on its level of total cozymase (Table 1). Prompt fixation after applying electrical pulses revealed no change in cozymase level. These experiments (Table 1, *B*) employed conditions under which glycolysis and other aspects of carbohydrate metabolism had been shown to undergo rapid change (Heald, 1956; McIlwain & Tresize, 1956).

Fig. 3. Cozymase of guinea-pig cerebral cortex during incubation in glucose-phosphate saline, aerobically or anaerobically as indicated. ○, Tissue without nicotinamide; ●, tissue with nicotinamide (mM).

2,4-Dinitrophenol in concentrations which greatly increased respiration was without effect on the cozymase level.

Anaerobic conditions, however, even in presence of glucose, led to fall in the tissue cozymase to half its original value (Fig. 3). The fall was not affected by 60 mM nicotinamide (the relatively high concentration that inhibits degradation of cozymase by ground cerebral tissues), nor by mM nicotinamide, nicotinic acid or adenosine 5-phosphate, added as potential precursors of cozymase. During a subsequent aerobic period, cozymase was maintained at the same lowered level, without resynthesis (Fig. 3) even when the compounds just mentioned were also present. The decreased level of cozymase was also relatively stable anaerobically when experimental conditions were changed. Thus when anaerobic glycolysis was decreased to some 30 % of its normal value, by addition of potassium salts or by electrical pulses (Table 1, *C* and *E*), cozymase changed little. Pulses applied throughout the period of anaerobic metabolism greatly lowered glycolysis, but decreased cozymase by only 15 %.

DISCUSSION

The two cerebral constituents now studied present an interesting contrast. Tissue ascorbate fell promptly in the absence of an external supply; with supply at about blood level it rose to concentrations approaching those normal to the brain, in 30–60 min. Restoration of ascorbate *in vitro* was thus slower than that of the tissue creatine phosphate but faster than that of tissue glycogen (McIlwain, 1955). The restoration was probably energy-assisted, as it

Table 1. Cozymase of slices of guinea-pig cerebral cortex

Cozymase values are the mean of duplicate determinations agreeing within 7%. Respiration (R) and glycolysis values (G) are averages for the slices from the different positions during the period just before their removal for fixing; they had typical, stable values during the experiments except when additions were made or pulses applied.

Treatment	Terminal respiration (μmoles of O ₂ /g./hr.) or glycolysis (μmoles of lactate/g./hr.)	Terminal cozymase (μm-moles/g. of fresh tissue) in slices cut in the following order:*		
		1st	2nd	3rd
A: 70 min. in glucose-phosphate saline; O ₂	R, 62	126	101	104
As A, with 0.2 mM-2,4-dinitrophenol in last 40 min.	R, 110	121	106	101
B: 40 min. in glucose-phosphate saline; O ₂	—	110	108	99
As B, with electrical pulses during last 20 min.	—	105	99	107
C: 70 min. in glucose-bicarbonate saline; N ₂ + CO ₂	G, 181	77	94	85
As C, with electrical pulses during last 40 min.	G, 50	81	87	81
D: as C	G, 168	94	87	83
As D, with electrical pulses during whole period	G, 29	82	75	67
E: as C	G, 152	99	91	82
As E, with 30 mM-KCl during last 40 min.	G, 42	99	80	85

* Slices cut first were those nearest to the outer surface of the brain; those cut third were nearest to the lateral ventricle.

did not take place in the absence of glucose and oxygen. In rats *in vivo*, injection of dehydro-ascorbic 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 μ moles/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 μ M, 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.