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BRAIN TISSUE AND FLUOROCITRATE SYNTHESIS

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Abstract—It is clearly very important to find the reasons for a discrepancy between experimental results *in vivo* and *in vitro*. Because of this and of our interest in the genesis of convulsions due to fluoroacetate poisoning, we have been forced to find reasons for the failure of brain tissue from the rat, pigeon and dog to synthesize fluorocitrate *in vitro*. No experimental support was obtained for the suggestion that this depended on the use of ATP instead of ADP. The failure was not due to failure to reach active centres. We found that it was due to the relatively small amount and slow action of acetyl CoA synthetase, leading to inactivity of the tissue before adequate synthesis *in vitro* could be obtained. The implications of the work are discussed.

We have found repeatedly [1, 2] that mitochondrial preparations from rat and pigeon brain tissue differ from some other tissues in that these do not appear to form fluorocitrate from fluoroacetate. In [1] pigeon brain tissue was used to estimate the fluorocitrate formed by guinea-pig kidney tissue from fluoroacetate. This problem may seem to be trivial; but it is a fundamental point bearing upon the genesis of the convulsions *in vivo*; and hence also upon the question of how far these events *in vitro* can be applied to explain events *in vivo*. We think that we have now found the reasons for the discrepancy.

EXPERIMENTAL

AnalaR reagents were used where possible. ATP, ADP, Na malate and Na pyruvate were supplied by Boehringer. Radioactively labelled materials were supplied by the Radiochemical Centre, Amersham. The Na fluorocitrate used throughout was a synthetic specimen, which may be assumed to contain 10·6 per cent of the active isomer. Mitochondria were prepared as given in [3]. Details of media are given below the tables.

It was suggested recently [4] that the failure to observe synthesis of fluorocitrate *in vitro* was due to a reinforcement of the homogenate with ATP rather than with ADP, i.e. to a biochemical artefact. We have therefore compared the effects of ATP and ADP using our technique [2], in which citrate accumulation in rat or pigeon brain tissue is used as an index of the formation of fluorocitrate. We found no difference within experimental error in experiments with mitochondrial and whole brain particles, and in one case with slices when we used reinforcement with either ATP or ADP. Hence the suggestion was not confirmed.

We turned therefore to the possibility that the brain tissue was relatively deficient in acetyl CoA synthetase, unlike other tissues [5]. It is well known [6] that fluoroacetate forms fluorocitrate via fluoroacetyl CoA. Using the method of Brand *et al.* [7] with ^{14}C-labelled acetate and sucrose, we proved that under our conditions acetate reached the inside of the brain mitochondria. This is consistent with experiments reported by Quastel and colleagues [8, 9]. From the compartmentation experiments of others [10, 11] it can be deduced that fluoroacetate also enters mitochondrial centres.

In order to test for the presence of acetyl CoA synthetase in the mitochondrial brain particles, we added acetate and estimated the extra formation of citrate as given by a block of citrate metabolism with fluorocitrate; in this, one assumes that an addition of malate will provide sufficient oxaloacetate to combine with the C_2 fragment via the citrate synthetase. Citrate was estimated chemically; though we realized that at these low concentrations, the error is apt to be large, we felt that the results would be more trustworthy in this case.

Table 1 shows the results. The number of estimations upon which the figures are based is also shown ($\pm 2\epsilon$), twice the standard error of the mean, for Exp. 2 and 3, except in two instances in Exp. 3, where flasks were lost. As the particles were not washed, Exp. 1 was a control. It showed that there was no

Table 1. Formation of citrate by rat brain mitochondrial particles, with or without addition of acetate, incubated for 35 min at 37°

		Additions	Citrate (μg)	
Exp. 1	A	None	61·1	(4)
	B	Acetate	63·1	(3)
	C	Fluorocitrate	67·2	(4)
	D	Acetate + fluorocitrate	65·8	(4)
Exp. 2	A	Malate alone	91·2 ± 9·24	(4)
	B	Malate + acetate	121·0 ± 5·62	(4)
	C	Malate + fluorocitrate	379 ± 46·98	(4)
	D	Malate + acetate + fluorocitrate	288 ± 37·28	(4)
Exp. 3	A	Malate alone	69	(3)
	B	Malate + acetate	57	(2)
	C	Malate + fluorocitrate	223 ± 59·84	(4)
	D	Malate + acetate + fluorocitrate	239 ± 32·52	(4)

Medium: Each flask contained in 3 ml: brain particles equivalent to 0·25 g original tissue; 20 mM Tris 7·4; 3 mM $MgCl_2$; 3 mM KH_2PO_4; 80 mM KCl; 3 mM ATP; 1 mM ADP; 0·5 μg/ml CoA. With additions where indicated of 1 mM Na malate; 10 mM Na acetate; 0·1 mM Na fluorocitrate.

Citrates estimated by colorimetric method [17].

Number of determinations (for calculation) in brackets.

Average figures given $\pm 2\epsilon$, ϵ = standard error of mean was calculated from four estimations.

need to take into account any residual activation of acetate. Experiments 2 and 3 were identical, using malate as a source of C_4. They show the effects of adding acetate alone and of this with a fluorocitrate block. The two experiments cover the kind of variations which can be expected. In Exp. 2, there was a slight increase found in citrate with adding acetate alone; this was barely significant, and was not seen with the fluorocitrate block. In Exp. 3, there was a small decrease with acetate alone, but no increase on adding fluorocitrate. It should be added that in numerous previous experiments, where pyruvate was used, much larger increases are found with additions of fluorocitrate. We interpret these results to mean that any effects induced by acetate are small if present.

The failure to see any substantial activation of acetate *in vitro* in rat brain tissue suggests that the tissue is very poorly endowed with acetyl CoA synthetase. This agrees with work on acetyl choline acetylation [12]. Since our experiments were completed, this has also now been found in different experiments by Knowles *et al.* [13], whose figures for rat brain give only 10·1 per cent of the synthetase in liver.

As will be seen in [1], it was still possible that the brain of the dog was better endowed with acetyl CoA synthetase. This animal shows severe convulsions on poisoning with fluoroacetate and is 100 times more sensitive (LD_{50} 0·05 mg/kg) to the poison than the rat. We have therefore done one experiment with a dog's brain to see whether it converts fluoroacetate to fluorocitrate activity. For this purpose we used our standard technique with pyruvate supplemented with malate. Table 2 shows that the dog's brain tissue behaved *in vitro* in a similar way to that from the rat and pigeon. With the cerebral cortex, fluoroacetate gave no extra citrate; the increase given by the thalamus tissue was barely significant. The sensitivity of the dog to the poison *in vivo*, therefore, is not due to an increased capacity to synthesize fluorocitrate.

There is still left in this field an outstanding problem, which requires explanation. Hendershot and Chenoweth [14] injected fluoroacetate into an isolated sector of the brain cortex of a dog *in vivo*, and recorded after a time interval of up to 135 min convulsive discharges as well as an accumulation of citrate. The important point was evidently that in their preparation the tissue remained functional for well over 1 hr. This would give time for the action of the small amount of acetyl CoA synthetase present to convert some of the fluoroacetate injected into fluorocitrate. In the ordinary homogenate, unlike brain slices or brain brei, tissue activity largely disappears within 20–30 min, which leaves little time for synthesis of fluorocitrate. Another point favouring the synthesis of fluorocitrate in their experiments was evidently that the fluoroacetate injected remained in their isolated piece of cortex. In this connection it may be recalled that more than 85 per cent of a dose of fluoroacetate injected into the subarachnoid space of a rat (under ether) left the brain within 1 min [15]. (This experimental fact raises the question as to the origin of fluorocitrate reported to be present in rat brain after exposure of a rat *in vivo* to an i.p. dose of fluoroacetate for 2 hr [16]).

Table 2. Dog brain tissue. Citrate present per 0·25 g particles after incubation at 37° for 30 min

Tissue	Additions	Citrate (μg/flask)
Cerebral cortex	None	71·8 ± 6·02
	Fluoroacetate	75·9 ± 5·41
Thalamus	None	57·9 ± 6·28
	Fluoroacetate	72 ± 7·10

Medium: Each flask contained in 3 ml: brain particles equivalent to 0·25 g original tissue; 20 mM Tris 7·4; 3 mM $MgCl_2$; 3 mM KH_2PO_4; 80 mM KCl; 3 mM ATP. 1 mM ADP: 1 mM Na malate: 2 mM K pyruvate ±1 mM Na fluoroacetate. Average figures given ± 2ε.

The male dog had a benign tumor of the left leg and was killed with Nembutal.

When fluorocitrate was added to the cerebral cortex, a rise in citrate of 60 μg/flask was observed, showing that citrate could be formed.

We thought that it might be possible to prove synthesis of fluorocitrate directly by using slices. In this preparation, it is well known that many of the tissue biochemical reactions remain stable for at least 2 hr at 37° in an O_2/CO_2 atmosphere. We are not recording these results in detail. After removal of proteins with trichloroacetic acid, extraction of any possible fluorocitric acid with ether and methylation, we were not successful in detecting any fluorocitrate by gas chromatography. But calculation showed that the minimum amount necessary to give convulsions was just below the amount detectable (10 μg/2·5 g tissue). Since results obtained by others [11] make it clear that the only explanation is that fluorocitrate must be formed, the experiment with slices really confirms Morselli *et al.* [15] in showing how little fluorocitrate injected into the subarachnoid space is needed to induce lethal convulsions in rats. They found that 0·5 μg killed all 200-g rats.

DISCUSSION

We are now satisfied that the failure to detect synthesis of fluorocitrate in brain tissue *in vitro* can be explained as ultimately due to the relatively small concentration of acetyl CoA synthetase in the tissue. It would be interesting to understand the biological reason for this. One speculative interpretation is that it is due to the importance of avoiding a possible concentration of the toxic acetaldehyde. In any case it is clear that when a tissue only metabolizes some compound slowly, there will be a discrepancy between events *in vivo* and *in vitro* if metabolism is too slow to take place before activity is lost in a homogenate.

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