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THE EFFECT OF INJECTIONS OF FLUOROCITRATE INTO THE BRAINS OF RATS

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Abstract—Intracerebral injection of fluoroacetate and fluorocitrate were made in rats by manual and stereotaxic technique. Fluorocitrate was found to be at least 100 times more toxic than fluoroacetate under various experimental conditions.

Fluorocitrate was more toxic when injected in the third cerebral ventricle, or in the nucleus reuniens thalami than when injected in the lobus frontalis.

Gas chromatographic determination of fluoroacetate shows that this compound is rapidly removed as such from the brain.

These results and their implications are discussed in relation to current views of the action of these compounds.

THE PROBLEMS of the actual toxicity of fluorocitrate to brain tissue are of importance for several reasons.

There is the fundamental question whether the convulsions caused by i.p. injection of fluoroacetate are caused by fluorocitrate synthesized locally in the brain or whether they are due to fluorocitrate carried to the brain after synthesis elsewhere in the body. Since fluorocitrate in small amounts blocks the enzyme aconitase, the amount of fluorocitrate leading to convulsions and death is very small; it follows that brain tissue appears to have no alternative pathway to the "citric acid cycle" for the final stages of metabolism.

It is known that in some animals, the brain tissue *in vitro* does not convert fluoroacetate into fluorocitrate. In the pigeon for instance homogenates of the brain can be used to estimate fluorocitrate in the presence of fluoroacetate[1,2] and in the rat, there is no apparent synthesis of a "citrate" inhibitor from fluoroacetate as shown by Peters and Shorthouse[3]. Qualitatively, it is known that intracerebral injections of fluorocitrate *in vivo* are much more toxic than injections of fluoroacetate. In the cat Prof. Szerb (personal communication) found that application to the brain surface of fluoroacetate (100 μg/ml) did not alter the electrical activity of the underlying cortex, whereas fluorocitrate (100 μg/ml) abolished all electrical activity within 15 min. In considering a possible local effect of fluoroacetate itself in cats, we must take into account the induction of "scissor-like" signs described in cats by Kelen and McEachern[4]; there are also the more recent observations by Lahiri and Quastel[5] on homogenates from rat brain, indicating that fluoroacetate in rather large concentrations (1·0 mM) can inhibit the glutamine synthetase and so alter ammonia metabolism.

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New techniques for the intracerebral injection of drugs[6] and for injection over specified areas of the brain gave the opportunity for more quantitative tests in the rat. We have also investigated the rate at which fluoroacetate can leave the brain.

MATERIALS AND METHODS

Sodium fluoroacetate (Monsanto) contained no measurable fluoride and was 95 per cent pure; the main impurity being sodium acetate (M. Shorthouse, personal communication). Sodium fluorocitrate was from the sample used by Ward and Peters[7] previously and was made from chemically synthesized triethyl-fluorocitrate. It was reckoned to contain 11·5 per cent of the biologically active isomer, as previously determined. In our experience, fluorocitrate remains stable for a long time if kept in the dessicator. The experiments of Table 2 were made with a more recent preparation.

Intracerebral injections

Intracerebral injections by the Valzelli technique[6] were made in a volume of 0·01–0·02 ml on female Sprague–Dawley rats anaesthetized with ether.

This technique takes advantage of the squamopetrosal fissure, immediately behind the zygomatic arch; injections by this technique reach the diencephalic zone. The stereotaxic technique is described below.

The solution of sodium fluorocitrate and of sodium fluoroacetate were made up with 20% gum arabic, which prevents a loss through the site of injection and which has been proved in this Institute to be innocuous.

Stereotaxic techniques

Sprague–Dawley adult female rats weighing 250 $\pm$ 10 g (brain weight 1·8 $\pm$ 0·1 g) were used.

Microinjections of sodium fluorocitrate or sodium fluoroacetate were made in different parts of the rat brain using a stereotaxic apparatus (C. H. Stoelting) under a light ether anesthesia.

The microinjections were made in the following brain centers: nucleus reuniens thalami (RE), nucleus rhomboideus thalami (RH), nucleus mediodorsalis thalami (MD), nucleus paraventricularis thalami (PV), nucleus medialis septi (MS), gyrus dentatus (FD), lobus frontalis (LF) and also in the third ventricle (V) (see Fig. 1).

The coordinates were calculated according to de Groot[8] (1959). The volume of the microinjection in all cases was 1 μl. As a control the same volume of pyrogen free distilled water was injected.

Gas chromatographic determination of fluoroacetate

Brain was homogenized with 75% alkaline ethanol (pH 7·5) in the ratio 1:10 (w/v) and then centrifuged at 15,000 r.p.m. $\times$ 30 min.

Fourteen ml of the supernatant were dried under vacuum. The residue was dissolved with 2 ml of water and then centrifuged for 10 min. at 10,000 r.p.m., 1·7 ml of the supernatant were frozen at $-20°$ and then dried under vacuum. The residue was dissolved with 0·05 ml of H_2SO_4, 40% (v/v) and then 0·05 ml of trifluoroacetic acid were added. The trifluoroacetic acid was used as an internal standard and kept in water at the concentration of 11·2 mg/ml (checked by a volumetric titration). One μl containing 5·6 μg of trifluoroacetic acid was injected directly in the chromatography column.

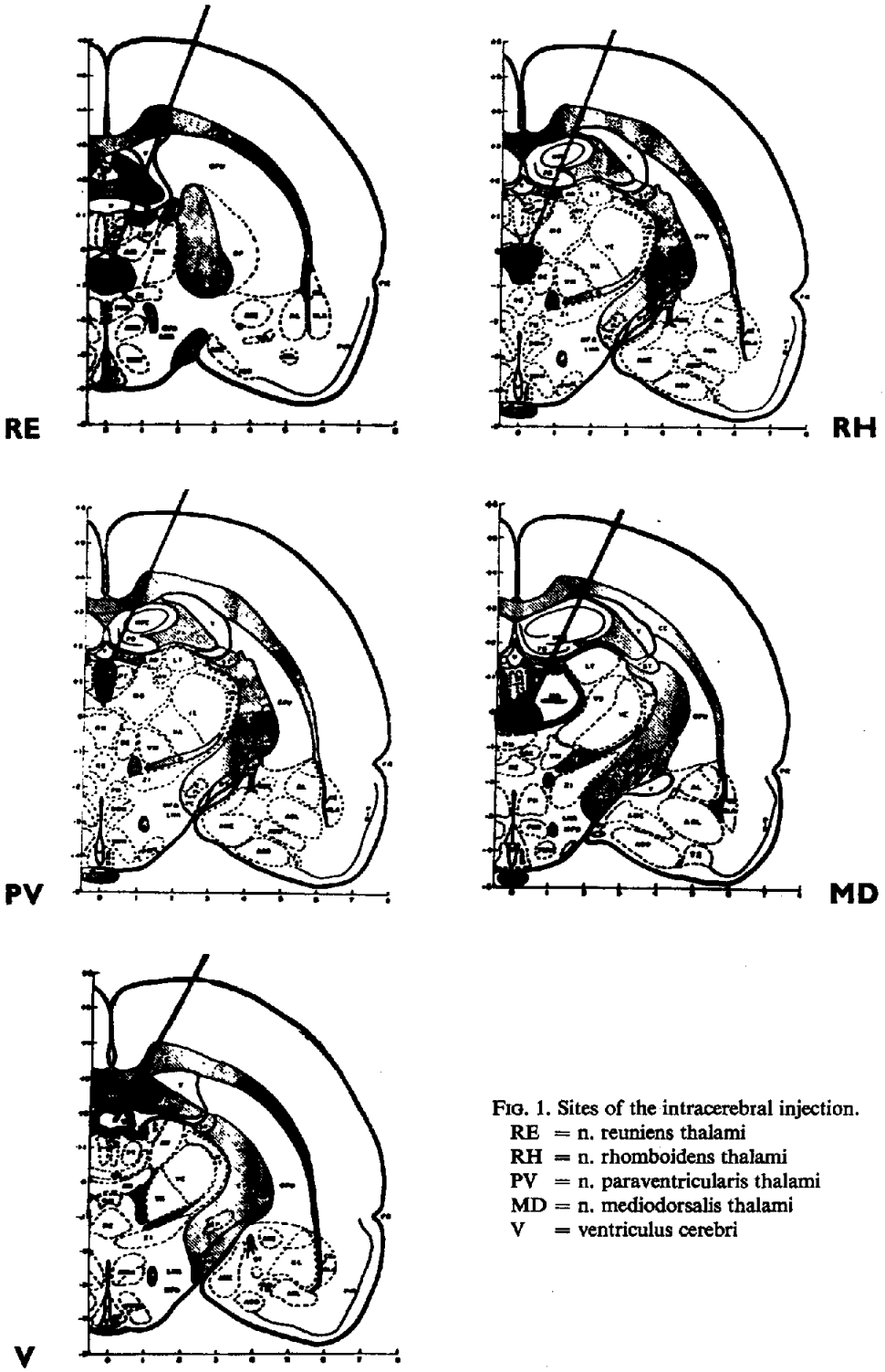

FIG. 1. Sites of the intracerebral injection.
RE = n. reuniens thalami
RH = n. rhomboidens thalami
PV = n. paraventricularis thalami
MD = n. mediodorsalis thalami
V = ventriculus cerebri

A 2 m glass column (i.d. 2 mm) was filled with Gas Chrom P (60–80 mesh) containing 1% phosphoric acid and 10% of LAC 446 (ethylene glycol adipate). Hydrogen was used as a carrier gas with a flow of 60 ml/min. The temperature was 130°.

Fluoroacetic acid was added at concentrations of 0·7, 1·4 and 2·8 μg/ml.

The surface of the peaks was calculated using the height × base at half height. A factor of correction was established as the ratio between a standard dose of trifluoroacetic acid and a standard of fluoroacetic acid. This ratio was in our experimental conditions 0·355. The recovery obtained by adding a standard dose of fluoroacetic acid to the brain was around 80 per cent.

RESULTS

1. *The effect of intracerebral fluorocitrate*

The results given in Table 1 and Fig. 2 which are confirmed for 10 μg of crude material in Table 2 prove that fluorocitrate is very toxic upon intracerebral injection.

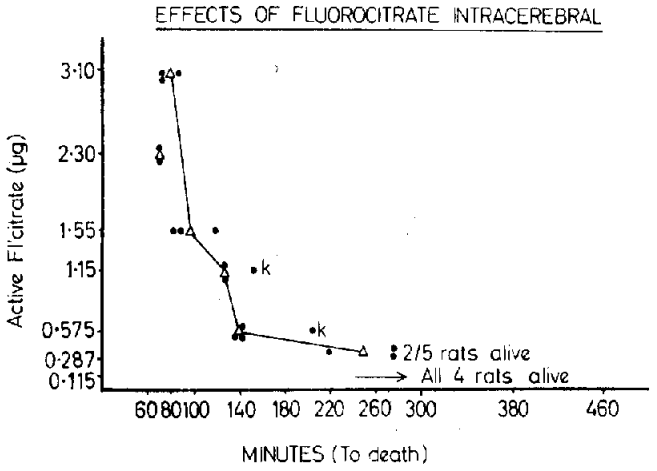

FIG. 2. Relation between dose and lethal effect of fluorocitrate (F citrate) injected by intracerebral route in rats. Time to death in min. K. killed in convulsions.

TABLE 1. INTRACEREBRAL TOXICITY OF SODIUM FLUOROCITRATE

Dose μg	Died	First convulsion average (min)
3·10	3/3	44
2·30	2/2	21
1·55	3/3	30
1·15	4/4	42
0·575	4/4	45
0·287	3/5	87
0·115	0/4	none

Note: (1) Weights (approx.) of rats for 2·30 μg, 250 g. for 0·287 μg, 134 g. Remainder 150 g.

(2) The dose given is that calculated for the active isomer which was 11·5 per cent of the crude fluorocitrate injected, i.e. 10 μg crude injected was equivalent to 1·15 μg active isomer.

All rats of 200 g were killed by 5 μg of the crude synthetic fluorocitrate, 2·5 μg killed 3/5. Since only 11·5 per cent of this preparation was the biologically active isomer, 5 μg is equivalent to 0·56 μg of the active sodium fluorocitrate isomer.

Reckoning the average weight of the rat brain as 1·40 g, the toxic brain dose per kilo wet weight is 0·4 mg, which does not allow for losses in other tissues. The quantitative result is surprisingly close to a theoretical estimate of 0·2 μg/g (2) required to block the citric acid cycle in pigeon's brain *in vivo*. It has been calculated (personal communication by Dr. K. C. Dixon) that fluorocitrate is as toxic as diphtheria toxin.

2. *The effect of fluorocitrate on different areas of the brain*

Data reported in Table 2 show that fluorocitrate injected intracerebrally is quite effective in producing convulsions. The concentration of 2·5 μg (corresponding to

TABLE 2. CONVULSANT ACTIVITY OF SODIUM FLUOROCITRATE INJECTED IN VARIOUS PARTS OF THE BRAIN (MIXED ISOMERS)

NaFC μg	Site of the injection	N. convulsant	N. deaths	N. convulsion in 2 hr	Remarks
—	MS	0/2	0/2	—	controls (1 μl/H_2O)
2·5	MS	2/4	0/4	15	—
5·0	MS	4/4	2/4	32	—
—	FD	0/2	0/2	—	controls (1 μl/H_2O)
2·5	FD	3/4	0/4	11	—
5·0	FD	4/4	2/4	20	—
—	LF	0/2	0/2	—	controls (1 μl/H_2O)
5·0	LF	0/4	0/4	0	—
10·0	LF	2/4	0/4	14	—
—	RE	0/2	0/2	—	—
1·0	RE	0/4	0/4	—	—
2·5	RE	4/4	1/4	28	—
5·0	RE	4/4	3/4	33	—
10·0	RE	3/3	3/3	30	—
20·0	RE	2/2	2/2	42	—
—	RH	0/1	0/1	—	controls (1 μl/H_2O)
2·5	RH	3/3	2/3	37	—
—	MD	0/1	0/1	—	controls (1 μl/H_2O)
2·5	MD	3/3	2/3	33	—
—	PV	0/1	0/1	—	controls (1 μl/H_2O)
2·5	PV	3/3	3/3	44	—
—	V	0/1	0/1	—	controls (1 μl/H_2O)
2·5	V	3/3	3/3	38	—
—	Midbrain	0/2	0/2	—	controls (1 μl/H_2O)
5·0	Midbrain	12/12	12/12	11	Valzelli's technique

MS : nucleus medialis septi
FD : gyrus dentatus
LF : lobus frontalis
RE : n. reuniens thalami
RH : n. rhomboideus thalami
MD : n. mediodorsalis thalami
PV : n. paraventricularis thalami
V : ventriculus cerebri
n = nucleus

about 0·25 μg of active isomer) produces long lasting convulsions after injection into almost all the parts of the brain assayed. However the injection of fluorocitrate into the lobus frontalis was convulsant only at the dose of 10 μg corresponding to about 1 μg of active isomer.

3. *The effect of intracerebral fluoroacetate*

From Table 3, it is seen that even a dose of 100 μg fluoroacetate injected intracerebrally did not kill any rat. This is at least 200 times the LD 100 of fluorocitrate. At the

TABLE 3. CONVULSANT ACTIVITY OF SODIUM FLUOROACETATE INJECTION INTO VARIOUS PARTS OF THE BRAIN

NaFaC μg	Site of injection	N. convulsant	N. deaths	N. convulsions in 2 hr	Remarks
—	V	0/2	0/2	—	controls (1 μg/H_2O)
50	V	5/6	2/6	19	—
25	V	4/6	0/6	16	—
10	V	2/6	0/6	3	—
—	RE	0/2	0/2	—	controls (1 μg/H_2O)
50	RE	6/6	4/6	13	—
25	RE	4/6	0/6	18	—
10	RE	2/6	0/6	2	—
—	Midbrain	0/4	0/4	—	controls (1 μg/H_2O)
50	Midbrain	0/6	0/6	—	Valzelli's Technique (1)
50	Midbrain	0/12	0/12	—	Valzelli's Technique (1)
75	Midbrain	3/6	0/6	3/6	Valzelli's Technique (1)
100	Midbrain	2/4	0/4	—	Valzelli's Technique (2)
100	Midbrain	2/4	0/4	—	Valzelli's Technique (2)
500	Midbrain	11/12	9/12	12	Valzelli's Technique

(1) (2) 150 g rats (2) All rats excited; 50 per cent not severe, clonic convulsions.
V = Ventriculus Cerebri. RE = n. Reuniens Thalami.

same time these experiments revealed a new point. Whereas doses of 50 μg had no effect, the larger doses of 100 μg induced temporary convulsions of a clonic type in 50 per cent of the rats. 500 μg killed all the animals with typical tonic and clonic convulsions.

Fluoroacetate injected in the ventricle or into the nucleus reuniens (see Table 3) was more toxic than when injected by Valzelli's technique. However even in the nucleus reuniens, fluoroacetate was about 100 times less toxic than fluorocitrate.

4. *Presence of fluoroacetate in brain*

Though these results appeared to be clear, it occurred to us that some estimate should be made of the time that the fluoroacetate remained in the brain after an injection. Accordingly, 150 g rats were given intracerebral doses of 100 μg/rat of sodium fluoroacetate in 10 μl; the animals were then killed 1, 15, 30 and 60 min after the treatment. Surprisingly, fluoroacetic acid was only present as such for 1 min after the injection; it was then less than 15 per cent of the amount injected.

DISCUSSION

Relative toxicity of fluorocitrate and fluoroacetate

The marked toxicity of fluorocitrate in the brain is very clear. From the injection into different areas it follows that the most sensitive parts of the rat brain were the n. reuniens thalami, the n. paraventricularis thalami and the ventriculus cerebri. Lethality was higher in the nucleus paraventralis thalami and also when the microinjections were made in the ventriculus of the brain. Intracerebral injection in the midbrain was similar, though faster in comparison with the microinjections.

The low dose of fluorocitrate, not more than 2×10^{-6}M, causing convulsion *in vivo* is in agreement with the effects found of inhibition of aconitase *in vitro* of 10^{-8}M (2); it is reasonable to conclude that the fluorocitrate is inhibiting the citric acid cycle in the brain at the point of the aconitase enzyme. It is indeed significant that this proves fatal, so that the brain appears to have no alternative pathway for obtaining the necessary energy to maintain its metabolism. Possibly there is a failure to synthesize ATP. It should be mentioned that, in the experience of one of us (R. A. Peters) and contrary to some statements, convulsions arising from i.p. or intracerebral doses of fluoroacetate and fluorocitrate have always been accompanied by a rise of citrate in brain tissue, consistent with a block in aconitase[2, 9].

Effect of fluoroacetate

In regard to the effect of fluoroacetate upon the brain, much of the indirect evidence points to the idea that the lethal convulsions induced by i.p. fluoroacetate injections are due to entry into the brain of very small amounts of fluorocitrate synthesized elsewhere. In favour of this, we have the failure to demonstrate conversion of fluoroacetate to fluocitrate by brain tissue *in vitro* and by inference in the cat brain *in vivo*.

At the same time, our experiments have demonstrated the new point that injections of fluoroacetate 50 μg into the midbrain (Valzelli technique) produced no ill effect, Although this is 100 times the dose of fluorocitrate which killed all animals, the larger dose of 500 μg in the midbrain was convulsive and lethal; this is best interpreted by the hypothesis that some of this large dose escaped from the brain and was converted in other tissues to fluorocitrate, some of which entered the brain; Dawson and Peters[10] noticed an escape of fluorocitrate in young rats, where a rise of citrate concentration was found in the kidneys after intracerebral injection.

The effect of fluoroacetate itself in the RE (thalamus) appears to be a genuine action of this compound. So far the only known enzymic effect of fluoroacetate itself is that on glutamine synthetase at a similar concentration *in vitro*[5]. It is possible that the excitation observed is induced by an interference with ammonia metabolism, though this is not yet proved *in vivo*. But the genuine action of the larger amounts of fluoroacetate may well point to a new interpretation of the experiments on the brain of the dog by Hendershot and Chenoweth[11], if they were observing a local effect of fluoroacetate and not of fluorocitrate synthesized from it. They used 12·5 μg/g tissue, and obtained some rises in citrate. It may be recalled that Guzman Barron and co-workers[12] considered that fluoroacetate interfered with the metabolism of acetate, a view confirmed by Quastel[13] in the presence of very small concentrations of acetate. The connection of this observation with those of ammonia metabolism is not yet clear,

though the phenomena seem to exclude the synthesis to fluorocitrate which has been prove to be blocked by acetate by Peters and Wakelin[1].

In conclusion, our studies show that fluorocitrate is at least 100 times more toxic in the brain than fluorocitrate. Direct measurements show that fluoroacetate as such leaves the brain very rapidly, unfortunately the amounts of fluorocitrate involved were too small to be measured. We do not know whether the fluoroacetate is removed from the brain by an unlikely conversion to another compound or whether it is carried away in the circulation. The latter possibility is supported by the known great vascularity of the brain (Cater *et al.*[14]).

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