Use of Fluorocitrate and Fluoroacetate in the Study of Brain Metabolism

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Abstract Fluoroacetate and its toxic metabolite fluorocitrate cause inhibition of aconitase. In brain tissue, both substances are preferentially taken up by glial cells and leads to inhibition of the glial TCA cycle. It is important to realise, however, that the glia-specificity of these compounds depends both on the dosage and on the model used. The glia-inhibitory effect of fluorocitrate as obtained by intracerebral microinjection in vivo is reversible within 24 h. A substantial inhibition of the glial TCA cycle by systemic administration of fluoroacetate requires a lethal dose.

Inhibition of the glial aconitase leads to accumulation of citrate and to a reduction in the formation of glutamine. Whereas the former is likely to be responsible for the main toxic effect of these compounds possibly by chelation of free calcium ions, it is the latter that has received most attention in the study of glial-neuronal interactions, since glutamine is an important precursor for transmitter glutamate and GABA.

Introduction Fluoroacetate and its toxic product fluorocitrate have recently received attention as specific inhibitors of glial metabolism. Fluoroacetate is the toxic ingredient of the South African poison plant Dichapetalum chymosum and of other Dichapetalum plants (Peters, 1963). It is also probably responsible for the neurological side effects of the anticancer drug 5-fluorouracil and its derivative fluoro-β-alanine (Koenig and Patel, 1971). Fluoroacetate was the first example of a substance undergoing "lethal synthesis," a term given by Peters (1952) to the conversion of a non-toxic compound to a toxic one, like fluorocitrate, in living tissue. Today fluorocitrate is regarded as a mechanism-formed substrate (Lauble et al., 1996).

Fluorocitrate and fluoroacetate can be administered in several different ways to achieve selective inhibition of glial metabolism. They can be used by intracerebral injection, by microdialysis of brain in vivo and during in vitro incubation of brain slices and cell cultures. Fluorocitrate can also be used systemically (Hassel et al., 1995; Largo et al., 1996; Paulsen et al., 1987; Paulsen and Fonnum, 1989; Stone and Joh, 1991; 1997; Swanson and Graham, 1994; Szerb and Issekutz, 1987). Generally fluorocitrate is 100–1,000 fold more active than fluoroacetate (Morselli et al., 1968; Swanson and Graham, 1994).

In this review we will first discuss the mechanism of action of fluorocitrate and fluoroacetate. Then we will discuss the evidence for obtaining a specific gliotoxic action with these compounds. We will also discuss the various factors that may contribute to the neurotoxic symptoms that accompany fluorocitrate poisoning. These fluorocompounds have been used extensively to study the importance of glial cells for nervous function. These are partly metabolic studies and partly studies to identify the physiological effects of inactivating glial cells. In conclusion we will summarise the various ways of using the fluorocompounds to inactivate glial cell function.

1 Fluorotoxin is used to describe both fluoroacetate and fluorocitrate.

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Mechanism and Action of Fluorotoxins

Molecular Mechanism of Fluorocitrate and Fluoroacetate

The conversion of fluoroacetate into fluorocitrate is analogous to the formation of citrate from acetate. Fluoroacetate is converted to fluoroacetyl CoA by acetate thiokinase in the presence of ATP and Mg\(^2+\). Fluoroacetyl CoA reacts with oxaloacetate in the presence of citrate synthase to give fluorocitrate (Peters, 1963). Fluorocitrate inhibits aconitase at very low concentrations (Peters, 1963). There are four isomers of fluorocitrate — of which the most active form is (2R, 3R)-2-fluorocitrate (erythro-2-fluorocitrate) (Stallings et al., 1980). Other halogenocitrates give similar, but much weaker, effects than fluorocitrate (Bosakowski and Levin, 1986).

It is a question whether fluorocitrate is a reversible or irreversible inhibitor of aconitase. Since fluoride ions are released in the reaction between fluorocitrate and aconitase (Tede and Casida, 1989; Villafranca and Platus, 1973), it has been suggested that fluorocitrate is a suicide substrate (Clarke, 1991). Recent experiments have shown that (-)-erythro-2-fluorocitrate loses F\(^-\) and is converted to 4-hydroxy-trans-aconitate, which binds tightly but not covalently to aconitase (Lauble et al., 1996). Other experiments have shown that the enzyme inhibition is reversed by Sephadex filtration and ammonium sulphate precipitation (Villafranca and Platus, 1973). This is consistent with the recovery of glutamine level or of the glial cell function in the brain 12–24 h after fluorocitrate treatment (Paulsen et al., 1987). It is also consistent with the necessity of continuous supply of fluorocitrate for studies lasting several hours. Experimentally one has therefore to treat fluorocitrate as a reversible inhibitor.

An inhibitory effect of fluorocitrate on the mitochondrial citrate carrier has also been proposed (Brand et al., 1973; Kirsten et al., 1978 Kun et al., 1977). This would interfere with the glial metabolism of citrate.

Glia-Specificity of the Fluorocompounds

In the early studies of brain metabolism it was observed that some radiolabeled substrates that are metabolized through the TCA cycle, e.g., glutamate, acetate, and bicarbonate, yielded a higher specific activity of glutamine than of glutamate. In contrast, others, such as glucose and lactate, gave a higher specific activity in glutamate than in glutamine. From these findings originated the concept of compartmentation of cerebral metabolism, involving at least two metabolically distinct TCA cycles. One TCA cycle metabolises acetate and is associated with a small pool of glutamate which is the precursor for glutamine. This compartment was termed the “small” compartment, as a reference to the size of the associated glutamate pool, and its TCA cycle was termed “synthetic” with reference to the synthesis of glutamine. The other TCA cycle is relatively inaccessible to acetate and is not associated with glutamine synthesis but avidly metabolises glucose. This was termed the large compartment (Berl and Clarke, 1969).

The small compartment was later identified as glial cells and the large compartment as neurons (Balazs et al., 1973; Martinez-Hernandez et al., 1977; Minchin and Beart, 1974). More recently, the strictly glial metabolism of externally added acetate was confirmed in vitro (Sonnewald et al., 1993) and in vivo (Hassel et al., 1992). Furthermore, a purely glial metabolism of externally added citrate has been demonstrated in vivo (Hassel et al., 1992).

Lahiri and Quastel (1963) were the first to show that fluorocitrate inhibited the incorporation of radioactivity from radiolabeled glutamate into glutamine, suggesting an effect on the small compartment. They proposed, however, that glutamine synthetase could be inhibited by the toxin. This was further investigated by Clarke et al. (1970), who showed that fluorocitrate and to lesser extent fluoroacetate inhibited preferentially citrate metabolism in the small glutamate compartment but did not have an effect on purified glutamine synthetase. Our interest in investigating fluorocitrate as a glio-toxic agent was further stimulated by the observation by Cheng et al. (1972) that fluorocitrate inhibited the formation of glutamine but not that of the transmitter acetylcholine. Since the synthesis of glutamine occurs only in glial cells and acetylcholine only in neurons, we took this as an indication of a possible specific glio-toxic effect.

Paulsen et al. (1987) demonstrated that intracerebral injection of 1 nmol fluorocitrate was accompanied by selective and reversible changes in glial cells only. The effect was maximal at 4 h and disappeared after 24–48 h. Ultrastructural investigation showed that astrocytes, particularly the end feet and to a lesser extent oligodendrocytes, were severely affected. The astrocytic cytoplasm appeared empty, and there was a loss of glycogen. The endoplasmatic reticulum was swollen and fragmented, and the mitochondria were swollen. A higher dose (2 nmol) also affected neuronal structures. Similar observations were obtained for the glial Müller cells in the retina after intravitreal injection of fluorocitrate (Virgili et al., 1991).

Biochemically, the effect of fluorocitrate on the endogenous levels of amino acids (Table 1) and on the synthesis of glutamine from different radioactive precursors is consistent with an action on the glial cells (Table 2). Intracerebral injection of fluorocitrate (1 nmol) caused a large loss of glutamine and glutathione and smaller changes in glutamate, aspartate and GABA (Paulsen et al., 1987). The alterations were maximal at 4 h after the injection and returned to normal by 12–24 h (Paulsen et al., 1987). Further, glutamine synthesis from 14C-labelled acetate was inhibited more than 95%, whereas the synthesis of glutamate from glucose was almost unaffected (Table 2; Hassel et al., 1992), indicating inhibition of the glial but not the neuronal TCA
cycle. We have also injected fluorocitrate twice with 24 h interval to see if this would increase the gliotoxic effect (Fig. 1). The effect on the levels and synthesis of amino acids was similar to the effects of a single injection. This is another indication that the effect of fluorocitrate is not irreversible.

The selectivity of fluorocitrate as a gliotoxin was confirmed by studying its effect on neuronal and glial cell cultures from cerebellum and cerebral cortex. Fluorocitrate (5–100 µM) more effectively inhibited the synthesis of glutamate and glutamine from [14C]glucose in cortical and cerebellar astrocytes than in cerebellar neurones (Hassel et al., 1995).

The selective glial metabolism of acetate and fluoracetate is probably due to a preferential uptake into glial cells and to the localisation of acetyl thiokinase in glia only as suggested by lesion studies (Sterri and Fonnum, 1980). As noted by Clarke (1991), however, the cellular localisation of acetyl thiokinase remains to be proven unambiguously. Since, exogeneous citrate is metabolized by glial cells only and there is no evidence for glial specific localization of citrate metabolising enzymes, we believe that the specific gliotoxic effect of fluorocitrate must be due to its specific uptake into glial cells.

Mechanism of Toxicity

Systemic administration of fluorocitrate may give cardic arrhythmia (Bosakowski and Levin, 1986). The main central nervous symptoms of acute intoxication by fluorocitrate and fluorocitrate are somnolence progressing to lethargy, followed by the development of convulsions indicating increased neuronal excitability (Bosakowski and Levin, 1986; Goldberg et al., 1966). The toxic effects should be explained from an inhibition of glial aconitase that results in a reduced carbon flux through the glial TCA cycle and a possible reduction in glial ATP production. Two significant biochemical changes observed are an increased extracellular level of citrate and a reduced synthesis of glutamine.

Inhibition of aconitase leads to accumulation of citrate in the brain and other organs (Buffa and Peters, 1986).
plausible that chelation of extracellular Ca\(^{2+}\) by the increased extracellular citrate may account for at least a part of the toxicity.

There are several examples that glutamine synthesis is more sensitive than ATP synthesis to inhibition by fluorocitrate. Swanson and Graham (1994) showed that fluorocitrate blocked the formation of glutamine in glial cell cultures from a medium containing glucose, but not from a medium containing 50 mM glutamate. Also, Benjamin and Verjee (1980) found that calcium administration increased the survival time of rats poisoned with fluorocitrate. It seems therefore plausible that chelation of extracellular Ca\(^{2+}\) by fluorocitrate may account for at least a part of the toxicity.

The reduction in glutamine synthesis has consequences both for ammonia fixation in the brain and for transmitter glutamate and GABA synthesis. Since glutamine synthesis is the main ammonia fixing reaction in the brain, there will be an increase in free ammonia (Hassel et al., 1995; Szerb and Redondo, 1993). Ammonia is a neurotoxin that can substitute for Na\(^+\) and K\(^+\), initially producing neuronal stimulation but eventually causing a depolarising block (Cass and Zieve, 1988). The reduction in glutamine, which is an important precursor for the transmitters GABA and glutamate, can explain the decreased release of these transmitters after prolonged treatment with fluorocitrate (Paulsen and Fonnum, 1989). This might also have contributed to the dysfunctional synaptic transmission in hippocampal slices treated with fluorotoxins (Berg-Johnsen et al., 1993; Keyser and Pellmar, 1994).

The reduction in the flux through the glial TCA cycle is expected to give a decrease in the ATP level of the glial cells. But Golberg et al. (1966) showed that 100 mg/kg fluorocitrate (a lethal dose) did not reduce the ATP level in the brain even through the citrate level had increased by 350%. As mentioned above there was no fall in ATP in brain slices treated with fluorocitrate (Benjamin and Verjee, 1980). Such findings do not support a decrease of ATP in glial cells as a primary effect. Further, there was no substantial fall in ATP in glial cell cultures incubated with fluorotoxins in the presence of glutamine (Hassel et al., 1994; Swanson and Graham, 1994). In contrast, Keyser and Pellmar (1994) found that treatment with fluorocitrate gave a substantial reduction in ATP in hippocampal slices during stimulation and in astroglial tumor cells, but not in synaptosomes. The ATP level and synaptic transmission, particularly the postsynaptic potential, were restored by addition of the glial substrate isocitrate (Keyser and Pellmar, 1994). During low activity of the glial cell the ATP level could be maintained by glycolysis (Swanson and Graham, 1994; Longuemare et al., 1994). In agreement with this notion, treatment with fluorocitrate in vivo caused a depletion of astrocytic glycogen stores (Paulsen et al., 1987). High synaptic activity during fluorocitrate exposure may lead to a decrease in glial ATP. This may have consequences for the maintenance of the low extracellular potassium level, the extracellular pH, and transmitter uptake (Largo et al., 1996).

APPLICATIONS OF FLUOROCITRATE AND FLUORACETATE AS GLIAL TOXIC AGENTS

Several scientists have taken advantage of the possibility that fluorocitrate is a specific gliotoxic agent and used this to identify specific properties of the glial cell. It should be kept in mind during the subsequent discussion that the compound are specific for glial cells during a narrow concentration range and that some index of glial and neuronal activity should be monitored in the investigation. In the metabolic section some new information are added on the metabolism of amino acids. In the physiological section we have tried to assess whether the conclusions drawn are based on critical use of the fluorocompounds.

**Metabolic Studies**

Fluorocitrate has been used with great success to study the metabolism and trafficking of substrates between glial cells and neurons. By intrastriatal injection of fluorocitrate a part of brain tissue is rendered devoid of metabolically active glial cells. The synthesis of the glial specific substrate glutamine is severely reduced from all precursors (Table 2). In addition the metabolism of acetate, a glial specific substrate, was markedly reduced, whereas there was only minor effects on glucose metabolism (Hassel et al., 1992; Table 2). The results show further that the conversion of [1,5-\(^{14}\)C]citrate and [U-\(^{14}\)C]2-oxoglutarate (Tables 1, 2) into radioactive glutamate and glutamine was reduced.
to the same level as with acetate. They are therefore glial selective substrates and cannot be used for trafficking from glial cells to neurons as previously suggested (Shank and Campbell, 1984; Sonnewald et al., 1994). Further, [U-14C]2-oxoglutarate yielded an increased labelling in aspartate and a decreased labelling of glutamate during fluorocitrate treatment. This is in accordance with the view that 2-oxoglutarate is fed directly into the glial TCA cycle to give oxaloacetate and subsequently aspartate (Table 1). There is not enough 2-oxoglutarate available to be used for glutamate synthesis. On the other hand, methionine sulfoximine that inhibits glutamine synthetase, but not the TCA cycle, allows 2-oxoglutarate to be converted to glutamate instead of being only fed into the TCA cycle (Table 1). Both fluorocitrate and methionine sulfoximine reduce the synthesis of GABA which depends on glutamine as a precursor.

We have extended the use of this model to include the metabolism of 14C-labelled aspartate and alanine (Table 3). During fluorocitrate treatment the incorporation of aspartate into glutamate was slightly reduced, but the incorporation into glutamine was seriously reduced. This shows that under normal conditions aspartate is taken up and metabolised both by glial cells and glutamatergic neurons in vivo. Under fluorocitrate aspartate is taken up and metabolised mainly by neurons and may substitute for glutamine as a source for glutamate. Because the level of alanine increases during fluorocitrate treatment (Table 1), the results with alanine are difficult to interpret. If the specific activity of alanine is reduced by fluorocitrate concomitant with the increase in unlabelled alanine, then the total amount of labelled glutamate formed is not reduced (Table 3). This means that alanine behaves as glucose and could be a substrate for neurons and glial cells as suggested previously (Hertz et al., 1992).

There has always been an open question whether a small amount of acetate could be metabolised by neurons. Recently we have shown by NMR-studies that a high dose of fluoroacetate (100 mg/kg) blocked completely the incorporation of [1,2-13C]acetate into glutamine, GABA, aspartate, and glutamate, whereas it only slightly decreased the labelling of GABA, aspartate, and glutamate from [1-13C]glucose. The effect of labelled acetate was not due to an inhibition of uptake, since labelled citrate from acetate was maintained at control level (Hassel et al., submitted). The NMR studies will also give us labelling of 13C into the different carbon atoms of glutamine and glutamate from [1-13C]glucose. From the C3/C4-enrichment of glutamine and glutamate during fluoroacetate treatment, it was possible to show that the turnover of transmitter glutamate was faster than the metabolic glutamate (Hassel et al., 1997). This is in agreement with the previous conclusions drawn from hypoglycaemic brains by Engelsen et al. (1986). They found that decortication, which remove glutamatergic striatal terminals, leads to lower turnover of glutamate in the striatum (i.e., in the nonglutamatergic striatum) than in an intact striatum.

Fluoroacetate and fluorocitrate have been used in brain slices experiments to study the importance of glial cells for nervous transmission or transmitter release. The results from these experiments have been difficult to evaluate since the fluorocompounds inhibited not only the glutamine pool for glutamate synthesis but also the uptake of neurotransmitters. In studies of hippocampal slices (Szerb and lissekutz, 1987) and of microdialysis of striatum (Paulsen et al., 1988, 1989) there were an increased extracellular level of glutamate during part of the fluorocompound treatment. This increase could be due to a less efficient glial uptake of transmitter glutamate or to an increased transmitter release due to a less efficient control of extracellular K+ (Largo et al., 1996).

Muir et al. (1986) and Lear and Ackerman (1990) have tried to use fluoroacetate as an indicator for glial cell metabolism in the same way as deoxyglucose is used to visualise cerebral metabolism. The turnover of fluoroacetate is, however, not rapid enough to be adopted for this purpose.

### Physiological Functions

An elegant example of the use of fluorocitrate as a glial toxin was seen in an investigation of induced cAMP synthesis following activation of adrenergic β-receptors in slices from cortex, striatum, and olfactory tubercle. Two hours incubation of brain slices with 0.1 mM fluorocitrate inhibited the cAMP synthesis by 75–95%. Neuronal function was maintained since the toxin did not have an effect on the electrophysiological parameters of the neurons in the slice (Stone et al., 1990). These findings were later confirmed by microdialysis with 1 mM-fluorocitrate (Stone et al., 1991). In microdialysis it is assumed that only 10 per cent of the compounds enters the tissue. These findings point to a
specific toxic effect by fluorocitrate on glial cells. The results have been confirmed by Suyama et al. (1994).

A similar procedure with incubation of brain slices with 1 mM fluorocitrate was used to show that the evoked cGMP response of atrial natriuretic peptide (ANP) was totally blocked, whereas the response of the C-type natriuretic peptide was only partially affected. As a control that the lesion was glial specific it was shown that the neuron specific cGMP response evoked by sodium nitroprusside, was only partially blocked. The exclusive glial localisation of ANP-response indicates that the receptor is expressed only by astrocytes (Goncalves et al., 1995).

The effect of fluorocitrate (0.1 mM) on the evoked field potentials (EPSP) in rat hippocampal slices have also been investigated (Berg-Johansen et al., 1993). The field-EPSP was markedly depressed after 7–8 h of superfusion with fluorocitrate. Control experiments using orthodromic stimulation indicated that this was a presynaptic rather than a postsynaptic effect. This presynaptic effect was believed to be due to lack of glutamine for glutamate synthesis. A control experiment using exogenously added glutamine was, however, not carried out. In similar experiments on hippocampal slice described by Keyser and Pellmar (1994), EPSP was restored by addition of the glial specific substrate isocitrate.

The important role of glial cells in maintaining synaptic transmission and ion homeostasis has been examined by microdialysis of the hippocampal CA1 area with 1 mM fluorocitrate. After 4 h, the astrocytes showed distinct morphological changes, whereas the neurons looked normal. At this time fluorocitrate dialysis caused a decrease of extracellular glutamine, blocked orthodromic transmission, and caused a moderate increase in extracellular K+ and lower pH. Spreading depression started between 4 and 8 h. At 8 h, the neurons showed signs of degeneration (Largo et al., 1996). The authors believed that spreading depression and neuronal degeneration were possibly a consequence of glial cell inactivation (Largo et al., 1996). Similar types of experiments were carried out by Szerb (1991). He also showed that 20 mM fluorocitrate increased spreading depression by elevating extracellular potassium. The early effects on pH and potassium homeostasis is clearly the result of an effect of fluorocitrate on glial cells. Since spreading depression is activated by a high extracellular potassium level, this can also, at least in part, be due to inactivation of glial cells.

Szerb and Redondo (1993) used fluorocitrate to study the fate of circulating ammonia in the brain. Microdialysis of fluorocitrate impaired the function of astrocytes and facilitated the entry of ammonia into the brain. Excitatory synaptic transmission was inhibited by the elevated brain ammonia and indicated that this elevation could be responsible for the CNS depression in hepatic encephalopathy. The depression of CNS outlasted the ammonia peak and could indicate a direct effect of fluorocitrate on neurones in this experiment.

Imamura et al. (1993) used 20 ml 10 mM fluorocitrate (0.2 nmol) injected into the visual cortex of a kitten to study the importance of glial cells for the shift of ocular dominance of visual cells following monocular deprivation for 3 days. Although the shift was retarded, it is questionable whether such small amounts would give rise to specific and long-lasting glial effects. Fluorocitrate (0.1 mM) and fluoroacetate (0.2 mM) have also been injected into the brain hemispheres to study the importance of astrocyte oxidative metabolism for memory processes in 1-day-old chicks (O'Dowd et al., 1994). The concentrations used and the time factors involved (minutes to achieve effects) raises the question whether the observed effects are glia specific in avians under these conditions.

**CONCLUSIONS**

The effect and the glia specificity of the fluorotoxins depend on the type of brain tissue and the activity of the tissue. Intracerebral injections of 1 nmol fluorocitrate lead to a part of brain tissue devoid of glial cell activity for several hours. Such preparations have been used for metabolic studies with considerable success.

Systemic administration of high doses of fluoroacetate (100 mg/kg) may be used for a limit time interval (less than 1 h). The dose is lethal and fluoroacetate administered systemically is more toxic to other organs like heart and kidney than to brain (Corsi and Granada, 1967). Lower doses of fluoroacetate have little effect on brain metabolism. Fluorocitrate cannot be used systemically.

Both fluorocitrate and fluoroacetate have been used with success in microdialysis experiments in brain in vivo. The concentrations used are for fluorocitrate 0.1–1 mM and for fluoroacetate 10–20 mM. This type of study can be used to evaluate the importance of glial cells in several different physiological experiments.

Both fluorocitrate and fluoroacetate have been used in vitro studies with brain slices or cell cultures. The concentrations used should be at the lower end of those selected for microdialysis. In studies of effects on synaptic transmission, it may be difficult to clearly differentiate which effects are due to inactive glial cell and which could be due to toxic effects on neurons.

The specificity of the fluorotoxins needs to be evaluated in every case. In metabolic studies the metabolism of labeled glucose and acetate can be used to assess the specificity of the toxic effects. When studying physiological functions, some ingenuity has to be used. As exemplified, certain glial and neuronal functions can be monitored (Stone et al., 1991). Alternatively, the addition of specific glial substrates such as isocitrate or 2-oxoglutarate should restore the glial functions (Keyser and Pellmar, 1994).

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