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DIFFERENTIAL TOXICITY OF FLUOROACETATE TO HEART, KIDNEY AND BRAIN MITOCHONDRIA OF THE LIVING RAT

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Abstract—Mitochondria were prepared from the heart, kidney and brain of rats intoxicated with fluoroacetate. Incubation with several substrates showed depression of respiration of heart mitochondria with pyruvate and succinate, and depression of respiration of kidney mitcohondria with pyruvate, succinate, citrate, β-oxobutyrate and L-glutamate; no inhibition of respiration was detected in brain mitochondria. Phosphorylative capacity, as expressed by the P:O ratio, was not affected in any of the tissues. However, a reduction of the respiratory control was observed in all cases.

IT HAS been shown[1–5] that fluoroacetate itself is not toxic: animal tissues convert it by enzymic synthesis (lethal synthesis) to fluorocitrate which has a blocking effect upon aconitase. However some features of the toxicity of fluoroacetate are still obscure. The brain tissue seems incapable to carry out the conversion to fluorocitrate, at least *in vitro*,[6] and the origin of the convulsions induced by fluoroacetate is not clearly understood. It was claimed by Fanshier *et al.*[7] that fluorocitrate, besides blocking aconitase, has an inhibitory effect on succinic dehydrogenase. Elliott and Phillips[8] showed that fluoroacetate interferes with glucose uptake into the cells: it was supposed that this effect is related to a fall in hexokinase activity.[9, 10]

The reaction of one organ may be different from that of another for reasons which are not entirely understood. The oxidation rate of some substrates was proved to be much reduced in mitochondria isolated from rats poisoned with fluoroacetate:[11] appreciable differences were exhibited by liver and skeletal muscle mitochondria. Specific differences were also exhibited by liver and kidney mitochondria upon addition of fluorocitrate *in vitro*.[7]

The experiments reported below were carried out with heart, kidney and brain mitochondria of rats treated with fluoroacetate. Some results with homogenates and slices are also described.

EXPERIMENTAL

Albino rats of the Wistar strain were injected with sodium fluoroacetate by intraperitoneal route (20 mg/kg body weight) and killed by decapitation 1 hr later. The animals were fed on a standard diet until 6 hr before the experiment.

Preparation of mitochondria

The heart ventricles were ground at 0° in a Lucite pestle homogenizer in 15 vol. of 0·24 M sucrose. The resulting suspension was centrifuged at 650 *g* for 3 min. The supernatant was recentrifuged at 7000 *g* for 10 min. The precipitate was dispersed in 0·24 M sucrose to a final concentration of 8–10 mg protein/ml.

The kidney cortex was ground in a Lucite pestle homogenizer in 9 vol. of 0·25 M sucrose containing 5 mM Na-EDTA* and 1 mM tris–HCl buffer, pH 7·4. The preparation was centrifuged at 800 *g* for 10 min. The supernatant was spun at 4000 *g* for 10 min. The precipitate was washed twice by suspending and centrifuging at 3000 *g* for 15 min and finally dispersed to a concentration of 15–20 mg protein/ml.

With brain, after several attempts with other methods, the procedure was adapted from the directions of Løvtrup and Zelander:[12] the mitochondrial preparation had little myelin contamination. The brain cortex from five or six animals was ground in a Teflon pestle homogenizer made by Kontess Glass Co. (Vineland, N.Y., U.S.A.) in 9 vol. of 0·44 M sucrose for 7 min. The suspension was centrifuged at 2000 *g* for 10 min and the supernatant was spun at 12,000 *g* for 10 min. The precipitate was washed three times by suspending and centrifuging at 7000 *g* for 12 min. With the aid of the Teflon pestle it was finally dispersed to a concentration of 5 or 6 mg protein/ml.

Incubation procedure

The incubation medium for heart mitochondria contained 13 mM phosphate buffer, pH 7·4: 10 mM tris–HCl buffer, pH 7·4; 13 mM NaF; 0·13 M sucrose; 25 mM glucose; 0·2 mM Na–AMP. Hexokinase (Sigma type III) was added with the AMP from the side arm of each flask at the end of equilibration. Incubation was carried out in a Warburg manometer in air at 25° for 20 min.

The medium for kidney mitochondria contained 15 mM phosphate buffer pH 7·4; 5 mM tris–HCl buffer, pH 7·4; 10 mM $MgCl_2$; 0·2 mM Na–EDTA; 40 mM sucrose; 40 mM glucose; 0·1 % bovine serum albumine; 1·5 mM Na–ATP. Hexokinase (Sigma type III) was added with the ATP from the side arm at the end of equilibration. The conditions of incubation were the same as for heart mitochondria.

The medium for brain mitochondria contained 12 mM phosphate buffer pH 7·4; 8 mM $MgSO_4$; 0·3 M sucrose; 25 mM glucose; 0·1 % bovine serum albumine; 1 mM K–ATP; hexokinase (Sigma type IV). The buffer was composed of potassium salts exclusively to avoid activation of cell membrane ATPase.[13] Incubation was carried out in air at 37°, usually for 20 min.

Pyruvate (with DL-malate), α-ketoglutarate, succinate, citrate, DL-β-oxobutyrate and L-glutamate were added as substrates to a final concentration of 15 mM. In some experiments lower concentrations were used: 5 mM or 2·5 mM. All salts were Na-salts for heart and kidney mitochondria, and K-salts for brain mitochondria.

Homogenates were incubated under the same conditions as mitochondria.

Incubation of slices of brain cortex was carried out in Krebs–Ringer solution with 13 mM phosphate (Umbreit *et al.*[14]) In some experiments 25 μM of unlabelled glucose and 0·5 μc of ^{14}C-universally labelled glucose were added to each flask. The oxygen uptake was measured in air at 37° for 3 hr.

* The abbreviations used are EDTA, ethylenediamine tetra-acetate; Tris, tris(hydroxymethyl)-aminomethane; ATP, adenosinetriphosphate; AMP, adenosinemonophosphate.

Analytical procedures

Nitrogen was determined by the micro-Kjeldahl technique, inorganic phosphate according to Fiske and SubbaRow,[15] citric acid by the method of Pucher *et al.*,[16] modified by Buffa and Peters.[2] $^{14}CO_2$ was collected in KOH, precipitated as $BaCO_3$ by adding $BaCl_2$, and counted at infinite thickness by using a conventional scaler with an end-window Geiger–Müller tube.

RESULTS

Effect of fluoroacetate poisoning upon heart mitochondria

The consumption of oxygen was appreciably reduced in the presence of 15 mM pyruvate or succinate (Table 1). Pyruvate respiration was rapidly inhibited: inhibition appeared about the same throughout the experiment. With succinate, inhibition was only slowly set up and appeared to rise progressively (Fig. 1). In the presence of

TABLE 1. THE EFFECT OF FLUOROACETATE POISONING ON RESPIRATION OF HEART MITOCHONDRIA

Substrate*	Mitochondria from normal rats		Mitochondria from poisoned rats		Percentage of change
	No. of expts	O_2 consumption (μl O_2/mg protein/hr)	No. of expts	O_2 consumption (μl O_2/mg protein/hr)	
Pyruvate (with malate)	9	121 (95–149)	8	38 (23–55)	−68
α-Ketoglutarate	4	77 (67–87)	3	76 (70–86)	
Succinate	4	55 (42–59)	3	35 (33–37)	−37
9-Oxobutyrate	4	7 (3–10)	3	5 (4–6)	
L-Glutamate	3	110 (90–135)	3	110 (103–115)	

The results represent mean values. Numbers in brackets represent the range of values recorded.
* Final concentration: 15 mM. Values for citrate were omitted because respiration was not appreciably stimulated by citrate in any case.

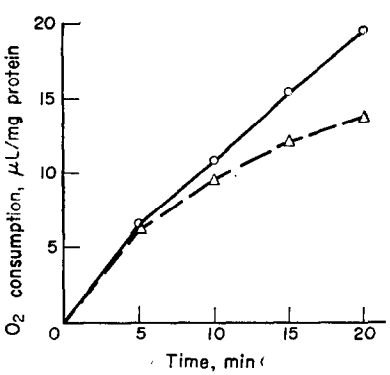

FIG. 1. Time-dependent inhibition of succinate respiration of heart mitochondria after fluoroacetate poisoning. . . . . : mitochondria from poisoned rats. ———: controls.

α-ketoglutarate, β-oxobutyrate and L-glutamate respiration was not reduced, also when substrates were added at a lower concentration (5 mM).

Oxidative phosphorylation was determined with pyruvate as substrate. The P/O ratio was not appreciably changed, whereas the respiratory control index (the ratio of the respiratory rate in the presence of added ADP to the rate obtained with no ADP added) was markedly reduced (Table 4).

Effect upon kidney mitochondria

O_2 consumption was reduced in the presence of 15 mM pyruvate, citrate, β-oxobutyrate or L-glutamate (Table 2): inhibition appeared fully established when the

TABLE 2. THE EFFECT OF FLUOROACETATE POISONING ON RESPIRATION OF KIDNEY MITOCHONDRIA

Substrate*	Mitochondria from normal rats		Mitochondria from poisoned rats		Percentage of change
	No. of expts	O_2 consumption (μl O_2/mg protein/hr)	No. of expts	O_2 consumption (μl O_2/mg protein/hr)	
Pyruvate (with malate)	9	77 (58–103)	10	32 (21–41)	−58
α-Ketoglutarate	4	95 (65–116)	5	82 (70–93)	
Succinate†	2	114 (109–119)	2	112 (108–116)	
Citrate	4	56 (51–63)	5	25 (22–29)	−56
β-Oxobutyrate	4	12 (5–19)	5	5 (3–7)	−60
L-Glutamate	3	65 (60–69)	3	44 (44–45)	−33

Values are calculated as for Table 1.
* Final concentration: 15 mM.
† In the presence of 5 mM or 2·5 mM succinate, respiration was reduced by 22 per cent.

first manometric reading was made, and did not appreciably change throughout the experiment. Succinate respiration was inhibited only at lower substrate levels (5 mM and 2·5 mM): inhibition amounted to 22 per cent (mean value of seven experiments). Respiration was not inhibited in the presence of α-ketoglutarate even at lower concentrations (5 mM and 2·5 mM).

Oxidative phosphorylation was determined with pyruvate as substrate. The P/O ratio was not significantly reduced, whereas the respiratory control index was appreciably reduced (Table 4).

Effect upon brain mitochondria

No effect was detected upon the rate of O_2 consumption in the presence of any substrate, even at lower concentrations (5 mM and 2·5 mM) (Table 3).

The P/O ratio in the presence of pyruvate was not appreciably changed. Only the respiratory control index was reduced (Table 4).

Also in homogenates of brain cortex, respiration was unaffected in the presence of any substrate.

In slices of brain cortex, respiration was equally unaffected, either in the absence or in the presence of glucose. No change was detected in the rate of glucose oxidation: [^{14}C] carbon dioxide produced from [^{14}C] glucose was about 0·1 μM/mg of protein after 3 hr incubations both of slices from intoxicated animals and of normal ones. The rate of oxidation was constant throughout the experiment.

TABLE 3. THE EFFECT OF FLUOROACETATE POISONING ON RESPIRATION OF BRAIN MITOCHONDRIA

Substrate*	Mitochondria from normal rats		Mitochondria from poisoned rats	
	No. of expts	O_2 consumption (μl O_2/mg protein/hr)	No. of expts	O_2 consumption (μl O_2/mg protein/hr)
Pyruvate (with malate)	10	136 (88–193)	9	130 (107–183)
α-Ketoglutarate	4	82 (61–100)	3	81 (71–89)
Succinate	4	119 (83–140)	3	122 (114–128)
Citrate	3	22 (21–23)	3	24 (22–27)
β-Oxobutyrate	4	25 (17–26)	3	22 (15–23)
L-Glutamate	3	140 (134–148)	3	130 (130–131)

Values are calculated as for Table 1.
* Final concentration: 15 mM.

TABLE 4. OXIDATIVE PHOSPHORYLATION AND RESPIRATORY CONTROL

Tissue	P:O		R.C.I.	
	Normal	Fluoroacetate	Normal	Fluoroacetate
Heart	2·61 ± 0·09	2·48 ± 0·49	4·2 ± 0·46	2·2 ± 0·46
Kidney	2·60 ± 0·10	2·44 ± 0·21	4·4 ± 0·14	2·5 ± 0·33
Brain	2·56 ± 0·04	2·46 ± 0·05	7·0 ± 0·93	4·0 ± 0·65

Citric acid was increased in freshly prepared brain homogenates from poisoned rats: in one experiment it amounted to 1·17 μg/mg protein, whereas a normal preparation contained 0·38 μg/mg protein. However citric acid accumulated during incubation at 37° in the presence of pyruvate in homogenates obtained both from normal and from poisoned animals. Fluoroacetate did not appear to produce any appreciable difference in citrate accumulation *in vitro*: after 30 min incubation, the citric acid level was about the same as in the normal control (2·47 and 2·78 μg/mg protein respectively).

DISCUSSION

The effect of fluoroacetate upon O_2 consumption with several substrates raises the question whether the rate-limiting role of inhibited aconitase can account for the rate of respiration in all cases, or inhibition of other enzymes may be involved. The

observation that succinate respiration is inhibited in kidney mitochondria at low substrate levels is in agreement with the results by Fanshier *et al.*[7] It may be noted that in heart mitochondria succinate respiration was inhibited also at higher substrate levels: however the initial rate was not affected. It is unclear whether the late inhibition of succinate respiration in heart mitochondria may be related to inhibition of succinic dehydrogenase by fluorocitrate, as it was claimed by Fanshier *et al.*[7] on the evidence provided by initial rate studies on kidney mitochondria.

On the other hand respiration of the heart and kidney mitochondria showed appreciable differences in response to fluoroacetate, reminiscent of the differences reported for mitochondria isolated from liver and skeletal muscle after fluoroacetate poisoning[11] and for liver and kidney mitochondria upon addition of fluorocitrate *in vitro*.[7] This seems difficult to reconcile with the view that the same biochemical mechanism is responsible for fluroacetate toxicity in all tissues.

Phosphorylation was never appreciably uncoupled. That is in agreement with previous observations by Buffa *et al.*[11] and Margreth and Azzone.[17] With regard to the lowering of the respiratory control index, it has been shown recently that addition of citrate *in vitro* results in swelling of mitochondria and it has been suggested that this effect is due to chelation of membrane-bound Mg^{2+} and increased permeability of the membrane to univalent cations: loss of respiratory control would ensue as a consequence of increased permeability.[18] It was reported by Baltscheffsky[19] that mitochondria incubated in the absence of magnesium may show loss of their respiratory control with no significant decrease of the P:O value. Dissociation of the two phenomena has been observed also in other experimental conditions.[20–22] The mechanism of this partial uncoupling (or "loose coupling") of phosphorylation was discussed at length by Azzone and co-workers[23] on the basis of the widely accepted hypothesis of energy-rich coupling intermediates. At present it appears that there are not sufficient data to decide whether the chemical coupling conception should be dismissed in favour of the chemiosmotic hypothesis proposed by Mitchell.[24, 25]

It is striking that no effect of the fluoroacetate poisoning could be detected in brain tissue *in vitro* (besides a reduction of the respiratory control index), in spite of the fact that the animals received a dose much larger than the LD_{50} and soon before being killed they were suffering excessively with signs in the nervous system. There were not even changes in citrate accumulation *in vitro*, to which more weight should be attached than to changes in oxygen uptake.[26, 27]

It was shown by Peters and Wakelin[26] that the injection of fluorocitrate into the subarachnoid space of pigeons is followed by death in convulsions and that addition of fluorocitrate to brain particles *in vitro* reduces the oxygen uptake in the presence of fumarate and pyruvate, whereas citrate accumulation is increased. Although fluoroacetate was not shown to be metabolized by brain preparations *in vitro*, it appears that condensation to fluorocitrate may be carried out by brain *in vivo*.[28] Fluorocitrate might also reach the brain from elsewhere.[29]

Whatever the case may be, it seems not unreasonable to suppose that fluorocitrate never reaches high concentrations in the nervous system of poisoned animals, even after large doses of fluoroacetate. That would explain the negative results of the *in vitro* experiments with nervous tissue preparations. Even if washing of mitochondria is avoided, diluting ten or twenty times in experiments with homogenates or slices would bring fluorocitrate below the effective level.

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