Conditions Affecting Inhibition of Tricarboxylic Acid Cycle by Fluoroacetate in Rat Liver Mitochondria.*† (25536)

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Earlier reports(1,2) indicated that fluoroacetate (FAc) poisoning in male rats did not lead to citrate accumulation in the liver, except when animals were castrated or exposed to X-ray irradiation(3). Various reasons(4, 5) were suggested for this lack of citrate accumulation. Experiments described here were undertaken to find out the response of rat liver mitochondria to fluoroacetate, and dependence of this response on various factors such as sex, nutritional state, X-radiation and 2:4-dinitrophenol.

Materials and methods. Wistar male rats, of 150 to 300 g weight maintained on Purina standard rat chow were used. *Starving conditions,* where used, entailed withdrawal of food 16-18 hours prior to experiment, with water furnished *ad lib.* In irradiation experiments animals received total body irradiation of 900 Roentgens at 30 r/minute. The characteristics of the machine were 250 kv, 15 milliamps with filters of 0.21 mm copper inherent, and 0.5 mm parabolic 1 mm aluminum. Irradiated animals were in groups of 6 and rotated 1 rpm. In all experiments, the animals were killed by cervical fracture and gross venesection. Livers, prior to excision, were immediately exposed and perfused *via* portal vein with about 60 ml of ice-cold 0.16 M KCl. Homogenization and/or fractionation of subcellular components adhered to methods described by Smith(6). Each Warburg flask contained 1 ml of basic medium composed of Phosphate pH 7.2 (0.06 M), $MgCl_2$ (0.006 M), and KCl (0.12 M). To this was added 1 ml of mitochondrial suspension in either 0.29 M mannitol or 0.25 M sucrose, with substrates added as indicated in specific experiments to give final volume of 3 ml. $Qo_2(N)$ values were determined by Warburg direct method at 37°C, with oxygen as the gas phase. All chemicals were of reagent grade. Fluorocitric acid (FC) was synthesized by the method of Rivett(7) and purified chromatographically using n-propanol, ammonia and water (6:3:1) (8). After air drying of chromatograms the acids were made visible by spraying(9) 2 test strips cut out 5 cm from opposite ends of streak. The fluorocitric acid preparation showed no trace of oxalic acid; however, it gave 3 distinct spots of unequal intensity. The lowest of these, Rf 0.32, corresponded to fluorocitrate as shown by comparison with enzymically formed fluorocitrate. This portion, representing 60-70% of original material, was now cut out as a strip parallel to starting line and eluted with distilled water. The eluates of several strips were pooled and lyophilized to 1 ml volume. Traces of ammonia and ammonium salt contained in this were removed by passage through a 1 x 15 cm Amberlite IR-120 column (hydrogen form). The free fluorocitric acid in the eluate was titrated with 0.1 N NaOH to pH 7.4 and lyophilized to dryness. The salt was kept in a vacuum desiccator over P_2O_5. A solution of this salt gave a single spot of Rf 0.32 to 0.35. ATP disodium salt was obtained from Pabst Labs, Milwaukee, Wisc. Testosterone and progesterone were purchased from Nutritional Biochemicals Corp., Cleveland, O. Nitrogen determinations were carried out by standard micro-Kjeldahl methods. Citrate was determined by the method employed by Brady, Mamoon and Stadtman(10), except that in our assays 0.3 ml of 5% (w/v) potassium permanganate was added dropwise and the color was developed with fresh solution of 5% (w/v) sodium sulfide.

Results. It was suggested(4) that absence of citrate accumulation in the liver of fluoro-

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TABLE I. Recovery of Citric Acid Following Decapitation or Freeze Killing of Animals.

	Citrate (μg/g wet tissue)	
	Decapitation	Liquid air freeze
Liver	31	32
Kidneys	725	810
No. animals	4	4

Animals were sacrificed 2 hr after intraper. administration of sodium fluoroacetate (5 mg/kg).

acetate poisoned rat was due to *in situ* destruction of this metabolite following sacrifice of the animal. To reexamine this latter point 2 groups of male rats were injected intraperitoneally with 5 mg/kg fluoroacetate. Two hours later one group was killed by decapitation, the liver and kidneys immediately removed and analyzed for citrate. The other group was killed by immersion of head in liquid air, and after exposing the viscera with a median incision, the whole animal was dropped in liquid air. Organs were thus immediately frozen solid. The frozen liver and kidneys were removed, powdered, and homogenized in ice-cold TCA. The results of citrate analyses (Table I) show that citrate content of livers of the 2 groups did not differ significantly. According to earlier observations (11) the liver of fluorocitrate poisoned rats does not destroy accumulated citrate to any significant degree.

Effects of starving on citrate accumulation and respiration in presence of fluoroinhibitors are shown in Table II. In starved animals there is a general increase of respiration. It is obvious that the inhibitory ratios, FAc/

TABLE II. Effect of Starving on Mitochondrial Respiration and Citrate Accumulation.

	Fed		Starved	
	Q_{O_2} (N)	Citrate,* μMole/N	Q_{O_2} (N)	Citrate,* μMole/N
Control	94	.36	187	.48
Fluoroacetate	74	1.94	143	1.66
Fluorocitrate	39	5.32	26	2.20
No. experiments	6		10	

Substrates and inhibitors in the flasks were: sodium fumarate (20 μMole), sodium pyruvate (9 μMole), ATP (.5 μMole), sodium fluoroacetate (3.3 μMole), synth. trisodium fluorocitrate (.27 μMole). 1 ml of mitochondria suspended in .29 M mannitol.

* Citrate values were calculated/mg mitochondrial nitrogen.

Control, are extremely close in both groups, namely 0.785 in fed and 0.763 in starved rats. Greater respiratory inhibition produced by fluorocitrate in the starved animal is not reflected in the citrate accumulation. The latter is in keeping with observations of others (12) who found that starvation reduced citrate accumulation in the liver of fluorocitrate poisoned rats. Investigation of conditions of inhibitory action of fluorocompounds is complicated by a time lag of 20-30 minutes before the effect on respiration of both homogenate and mitochondrial system becomes noticeable (Fig. 1). The appearance of respiratory in-

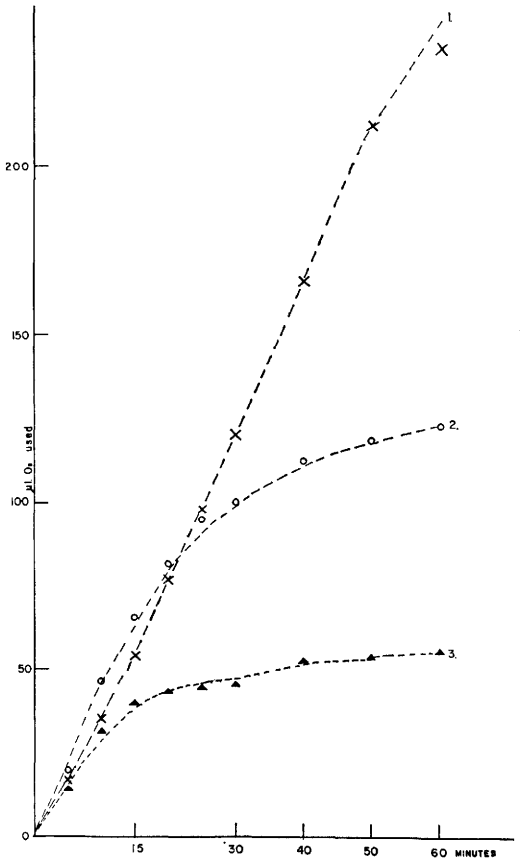

FIG. 1. Oxygen consumption by liver mitochondria obtained from starved rats. (1) Control. (2) Sodium fluoroacetate (1.1×10^{-3} M). (3) Synth. trisodium fluorocitrate (5×10^{-5} M). Substrates and conditions are as given in Table II.

hibition by 0.1 to 0.2 mM fluorocitrate will set in within the first 15 minutes.

Effect of sex and sex hormones. Reports of *in vivo* experiments do not completely agree

TABLE III. Effect of Sex and Sex Hormones on Respiration and Citrate Accumulation.

	Additions	Respiration Q_{O_2} (N) ♂	Respiration Q_{O_2} (N) ♀	Citrate, μMole/mg N ♂	Citrate, μMole/mg N ♀
a)	None	159* ±16†	217 ±20	.73 ± .13	1.19 ±.06
	FAc	163 ± 7	184 ±31	2.62 ± .50	2.36 ±.35
	T	122 ± 5.5	157 ±23	.53 ± .11	.99 ±.16
	" + FAc	104 ± 1	130 ±13	1.82 ± .41	2.73 ±.23
	P	108 ± 2	131 ±17	.55 ± .09	1.07 ±.09
	" + FAc	88 ± 2.5	91 ±10	1.19 ± .31	1.60 ±.05
b)	None	157 ± 2.5	262 ±42	1.78 ±1.09	4.31 ±.80
	FAc	159 ±25	263 ±12	2.10 ± .74	4.84 ±.56
	T	127 ± 5.5	208 ±23	1.53 ±1.04	3.46 ±.53
	" + FAc	128 ±13	227 ±22	2.16 ± .75	5.04 ±.26
	P	101 ± 5	132 ±20	.90 ± .50	1.47 ±.15
	" + FAc	100 ± 4.7	120 ± 8	1.27 ± .10	1.98 ±.11
No. animals		6	6	6	6

All flasks contained fumarate (40 μM), ATP-Na_2 (4 μM). Under b), sodium pyruvate (20 μM) was additionally given. Other additions as indicated: sodium fluoroacetate (FAc) 3.3 μM, testosterone (T) 1.6 μM, progesterone (P) 1.6 μM. Steroid solvent was prepared as described by Wade and Jones (17). Mitochondria were suspended in sucrose (.25 M).

* Mean. † Stand. error.

that there is a sex difference in response of rat liver to fluoroacetate poisoning. Our experiments were designed to study the *in vitro* existence of any such difference. Some results are given in Table III. Statistical comparison of the significance of differences both with respect to respiration and citrate accumulation between mitochondria of either sex revealed no difference with fumarate as substrate. In presence of fumarate the mitochondrial system of either sex was able to accumulate citrate; whence both sexes are able to convert fluoroacetate to fluorocitrate. However this effect is abolished by pyruvate. Pyruvate moreover leads to a significant increase of citrate accumulation in the female system only. Addition of sex hormones did not bring out any difference in response with respect to respiration or citrate accumulation. Addition of sex hormones alone did not lead to citrate accumulation. However progesterone prevented fluoroacetate-produced citrate accumulation to a significant degree in liver mitochondria of male rats, but only to a 5% level of fidelity in female rats. Progesterone alone or in combination with fluoroacetate inhibited respiration in both sexes.

Effect of irradiation. It was suggested(13) that the increased accumulation of citrate in the liver of irradiated male rats was due to effect of X-ray radiation on the metabolic pathways related to citrate oxidation which were specific to male rats. Table IV summarizes results obtained from a comparison of liver mitochondria of male rats of different nutritional states with those of rats exposed to irradiation. Statistical analysis of the results revealed that in presence of fluoroacetate citrate accumulation increased in the mitochondria of irradiated animals only in comparison with those of starved rats ($p<0.03$) but not with those of fed rats ($p = 0.05$). This agrees well with the *in vivo* findings(13).

Effects of 2:4-dinitrophenol and fluoroinhibitors. As a corollary to earlier studies on comparative effects of dinitrophenol (DNP) and the fluoroinhibitors(14,15) some values were obtained on citrate accumulation and respiration with fluoroinhibitors in presence of DNP (Table V). When DNP and fluorocompounds are simultaneously added to the system inhibition not only becomes greater with respect to respiration but to citrate accumulation as well. Judah and Rees(16) described the effect of DNP as protecting citrate oxidation and showed that it was due to failure of fluoroacetate activation through lack of ATP resynthesis. They gave no information on citrate accumulation, consequently it is uncertain whether the DNP concentration re-

TABLE IV. Effect of X-radiation on Citric Acid Levels of Rat Liver Mitochondria.

	Fed	Starved	Irradiated
FAc	340 ± 35*	241 ± 24	793 ± 213
No. animals	6	5	10

System as described in Table II.

* Mean ± stand. error.

TABLE V. Effect of 2:4-Dinitrophenol and Fluorocompounds on Mitochondrial Respiration and Citrate Accumulation.

Additions to system	Q_{O_2} (N)	Citrate, μMole/mg N
None	94	.07
DNP	120	.08
FAc	75	1.35
" + DNP	70	.92
FC	39	3.35
" + DNP	26	2.51
No. experiments	4	

Conditions and substrate concentrations were same as in Table II. DNP was .1 μMole/flask.

ported by them was sufficient to produce complete inhibition of fluoroacetate conversion to fluorocitrate. In our system we were unable to show a reactivation of respiration when DNP and fluoroacetate were incubated together, even at higher DNP concentrations.

Summary. In presence of fumarate, rat liver mitochondria responds with citrate accumulation to fluoroacetate poisoning. This response is not sex-dependent. There is a time lag to the inhibitory effect of fluorocompounds on respiration. Ratios of respiration of fluoroacetate and control system of starved and fed animals were in close agreement. Testosterone did not abolish the effect of fluoroacetate on citrate accumulation, whereas progesterone significantly reversed it only on liver mitochondria of male rats. Progesterone, like DNP, when in combination with fluoroacetate, inhibited respiration. In normal nutritional states irradiation does not significantly increase citrate accumulation in the liver mitochondria of male rats.

1. Buffa, P., Peters, R. A., *J. Physiol.,* 1949, v110, 488.

2. Potter, V. R., Busch, H., *Cancer Res.,* 1950, v10, 353.

3. Dubois, K. P., Cochran, K. W., Zerwic, M. M., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 452.

4. Beaulieu, M. M., Dallemagne, M. J., *Bull. Soc. Chim. Biol.,* 1953, v35, 969.

5. Busch, H., Potter, V. R., PROC. SOC. EXP. BIOL. AND MED., 1952, v80, 701.

6. Smith, R. E., *Ann. N. Y. Acad. Sci.,* 1956, v62, 403.

7. Rivett, D. E. A., *J. Chem. Soc.,* 1953, 3710.

8. Buffa, P., Peters, R. A., Wakelin, R. W., *Biochem. J.,* 1951, v48, 467.

9. Reid, R. L., Lederer, M., *ibid.,* 1951, v50, 60.

10. Brady, R. O., Mamoon, A. M., Stadtman, E. R., *J. Biol. Chem.,* 1956, v222, 795.

11. Gal, E. M., Peters, R. A., Wakelin, R. W., *Biochem. J.,* 1956, v64, 161.

12. Fawaz, E. N., Tutunji, B., Fawaz, G., PROC. SOC. EXP. BIOL. AND MED., 1956, v92, 311.

13. Ord, M. G., Stocken, L. A., *ibid.,* 1953, v83, 695.

14. Fairhurst, A. S., Smith, R. E., Gal, E. M., *Bioch. Pharmacol.,* 1959, v1, 273.

15. ———, *ibid.,* 1959, v1, 280.

16. Judah, J. D., Rees, K. R., *Biochem. J.,* 1953, v55, 664.

17. Wade, R., Jones, H. W., *J. Biol. Chem.,* 1956, v220, 547.

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Buffy Coat Cell Migration from Symmetrical Explants in Presence and Absence of Clotted Plasma.* (25537)

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Difficulties most commonly encountered in work concerned with buffy coat cell migration are (a) it is very difficult, if not impossible, to obtain symmetrical and reproducible explants using conventional technics, (b) migration is slow when cells are imbedded in clotted plasma. Our purpose was to develop a simple and reproducible procedure to obviate these difficulties.

The method consists essentially of filling small bore polyethylene tubing with material to be studied and then cutting it into small pieces handled as if they were tissue explants. Intradermic polyethylene tubing formulation PH-F-Adams PE-50† was sterilized by filling

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† Sterile tubing obtained from Clay Adams.