The Inhibition by Fluorocitrate of Rat Liver Mitochondrial and Extramitochondrial Aconitate Hydratase

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1. The effects of synthetic fluorocitrate were studied on: (a) the oxidation of citrate and *cis*-aconitate by rat liver mitochondria; (b) the activity of the aconitate hydratase found in the liver cell sap; (c) the activity of the aconitate hydratase solubilized from liver mitochondria. 2. Fluorocitrate was found to be a potent inhibitor of oxidation of citrate but only a weak inhibitor of oxidation of *cis*-aconitate: 6.7 μ M-fluorocitrate (containing 4% of the inhibitory isomer) caused 94% inhibition of the oxidation of citrate (2mM) whereas 1.0mM-fluorocitrate was necessary to provoke the same inhibition when *cis*-aconitate (2mM) was the substrate. The degree of inhibition varied in relation to the respiratory state of mitochondria when fluorocitrate was added. The inhibition could be partially reversed by *cis*-aconitate. 3. The aconitate hydratase extracted from the mitochondria was much less inhibited by fluorocitrate than was the mitochondria-bound enzyme, and the aconitate hydratase found in the cell sap was even less sensitive. 0.3mM-Fluorocitrate was required to cause 50% inhibition of the reaction citrate $\rightarrow$ *cis*-aconitate, catalysed by the aconitate hydratase extracted from the mitochondria, and 1.2M-fluorocitrate for the extramitochondrial enzyme. For both enzymes the reaction citrate $\rightarrow$ *cis*-aconitate was 2–3 times more sensitive to fluorocitrate than was the reaction isocitrate $\rightarrow$ *cis*-aconitate. The inhibition was of the competitive type for both reactions.

Lotspeich, Peters & Wilson (1952), Morrison & Peters (1954) and Peters (1961) found that soluble preparations of aconitate hydratase [citrate (isocitrate) hydrolyase, EC 4.2.1.3] were inhibited by fluorocitrate and that both the reactions catalysed by the enzyme were affected by the inhibitor. These observations were made on preparations obtained from pig heart muscle homogenates in buffered saline solutions and ranged from crude extracts to purified solutions of the enzyme.

Busch & Potter (1952) reported that in kidney homogenates obtained from rats poisoned with fluoroacetate (fluorocitrate) there was a block in oxidation of citrate, though not of *cis*-aconitate, and recent experiments in this laboratory on the biochemical effects of fluoroacetate poisoning in rat liver have confirmed this. Thus an apparent difference seemed to exist between the effects of fluorocitrate on aconitate hydratase *in vitro* and *in vivo*.

Dickman & Speyer (1954), using rat liver, and Shepherd, Li, Mason & Ziffren (1955), using human liver, showed that when the tissue is homogenized and fractionated by differential centrifugation a large proportion of the total aconitate hydratase activity is found in the cell sap, whereas

the rest of the activity is associated with the mitochondrial fraction. Dickman & Speyer (1954) showed also that the mitochondria-bound enzyme can be obtained in a soluble form by freezing and thawing the particles.

These observations raised the question of whether the cellular distribution of the enzyme could be responsible for the difference in inhibition of aconitate hydratase caused by fluorocitrate *in vitro* and *in vivo*. The effects of synthetic fluorocitrate have been investigated on oxidation of citrate and *cis*-aconitate by isolated rat liver mitochondria and on the conversion of citrate into *cis*-aconitate and of isocitrate into *cis*-aconitate by the aconitate hydratase of the soluble cytoplasm and by the enzyme solubilized from mitochondria. The effects of fluoroacetate and fluorocitrate poisoning of the rat on the different forms of liver aconitate hydratase have also been investigated.

MATERIALS AND METHODS

Animals. Young adult Wistar strain albino rats of both sexes fed on a standard diet and water *ad libitum* were used. The animals were killed by decapitation and bled.

Preparation of the liver cell sap. The liver was rapidly

excised and placed in 0.25M-sucrose at 0°; all subsequent manipulations were carried out at 0–2°. The tissue was homogenized in 10 vol. of 0.25M-sucrose in a glass homogenizer of the Potter–Elvehjem type with a tightly fitting Teflon pestle. Usually 5g. of tissue was homogenized by three complete excursions of the pestle in 60 sec., the pestle rotating at 1500 rev./min. The homogenate was centrifuged at 139000g_{av.} for 45 min. in a Spinco model L ultracentrifuge. The supernatant was carefully withdrawn with a syringe and used as such for aconitate hydratase measurements. These preparations could be stored frozen at –20° for at least 1 week without decrease in activity.

Isolation of liver mitochondria. The tissue homogenate, prepared as described above, was centrifuged at 900g for 10 min. The supernatant was centrifuged at 14500g for 10 min. The supernatant, together with the fluffy layer, was discarded, and the sediment was resuspended in 10 ml. of 0.25M-sucrose and centrifuged as before. The mitochondrial pellet was resuspended in 0.25M-sucrose to a final concentration of 35–60 mg. of mitochondrial protein/ml. Mitochondria so prepared were used for the metabolic experiments; those to be employed for the extraction of aconitate hydratase were washed twice with 0.25M-sucrose.

Extraction of aconitate hydratase from mitochondria. The freezing-and-thawing method of Dickman & Speyer (1954) was followed with minor modifications. The twice-washed mitochondrial pellet from 5g. of tissue was resuspended in 10 ml. of distilled water in a test tube and this was stored at –20° for 24 hr. The test tube was then left at room temperature until the suspension was thawed, but its temperature was not permitted to rise above 0°. In early experiments the suspension was centrifuged at 30000g for 30 min.; subsequently it was centrifuged at 139000g_{av.} for 45 min. The supernatant was carefully decanted and used as such. The second method yielded preparations of aconitate hydratase with higher specific activity. The addition of sucrose to 0.25M final concentration did not cause any change in activity of these preparations. The freezing-and-thawing method was used also to extract the aconitate hydratase activity from the fractions formed by cellular debris and nuclei referred to in Table 2.

Experiments in vivo. Rats were injected intraperitoneally with freshly prepared solutions containing 10 mg. of fluoroacetate/ml. or 40 mg. of fluorocitrate/ml. (Table 4); control rats were injected with 1.0 ml. of 0.9% NaCl/kg. body wt. The poisoning effects of fluoroacetate and fluorocitrate were indicated by a very marked decrease of the body temperature (Buffa & Filippini-Lera Buffa, 1959). After 60 min. the animals were killed by decapitation.

Analytical methods. The aconitate hydratase activity of the soluble preparations was measured by the method of Racker (1950) as described by Anfinsen (1955). The assays were carried out as described in the text; a Beckman DU spectrophotometer was used for the measurements. Cytochrome oxidase activity was measured with a Clark-type polarographic electrode in a model 53 oxygen monitor (Yellow Springs Instruments Co., Yellow Springs, Ohio, U.S.A.) connected to a Sargent SRL recorder. The reaction medium contained in 3.6 ml. final volume: 40 mM-sodium-potassium phosphate buffer, pH 7.4, 0.4 mM-AlCl₃, 0.1 M-cytochrome c, 11.5 mM-sodium ascorbate and 0.9–1.5 mg. of protein. The temperature was 38° and the reaction was started by adding the ascorbate. The oxygen uptake and the ADP/O ratio of isolated mitochondria were also

measured with a Clark-type polarographic electrode. The composition of the medium is given in the legend to Fig. 1. The respiratory states of mitochondria were defined according to Chance & Williams (1956).

The protein concentration of aconitate hydratase preparations was measured by the spectrophotometric method suggested by Anfinsen (1955); mitochondrial protein was determined by a micro-Kjeldahl procedure, using the factor 6.25 to convert nitrogen values into protein values.

Chemicals. All chemicals were of analytical grade. Trisodium DL-isocitrate, *cis*-aconitic acid and ADP (sodium salt) were products of Sigma Chemical Co. (St Louis, Mo., U.S.A.). Sodium fluoroacetate and synthetic trisodium fluorocitrate were kindly given by Sir Rudolph A. Peters, F.R.S.; the latter compound contained about 4% of the inhibitory isomer (personal communication from Sir Rudolph Peters) and was treated according to the procedure of Ward & Peters (1961). The fluorocitrate concentrations reported in this paper refer to total fluorocitrate rather than to the inhibitory isomer. When necessary, the reagents were adjusted to pH 7.4 with NaOH or HCl. Glass-distilled water was used throughout.

RESULTS

Effect of fluorocitrate on oxidation of citrate and *cis*-aconitate by mitochondria. Under our conditions (Fig. 1) added citrate and *cis*-aconitate were actively oxidized by isolated rat liver mitochondria. In 17 experiments the mean oxygen uptake with 2 mM-citrate as substrate for mitochondria in respiratory state 3 was 34.05 μ l. of oxygen/mg. of mitochondrial protein/hr., the respiratory control index was 4.67 and the ADP/O ratio was 2.31. Added *cis*-aconitate was oxidized at a rate about 30% higher than that for citrate.

The oxidation of citrate was inhibited by fluorocitrate and the degree of inhibition varied with the respiratory state of the mitochondria at the time of addition of fluorocitrate (Fig. 1). Maximum inhibition was observed when the inhibitor was added to mitochondria in respiratory state 1, i.e. before the substrate and ADP. State 4 respiration was slightly impaired but the inhibitory effect became fully apparent on addition of the phosphate acceptor (Fig. 1, curve I). When fluorocitrate was added to mitochondria in state 4, i.e. after the substrate and before ADP, it inhibited only state 3 respiration and the effect was moderate (Fig. 1, curve II). A somewhat similar degree of inhibition was observed on addition of fluorocitrate to mitochondria in state 3 (Fig. 1, curve III). Fig. 2 illustrates the relationship between the concentration of fluorocitrate added to mitochondria in state 1 and the inhibition of state 3 respiration.

The sensitivity to fluorocitrate of oxidation of citrate varied in different preparations of mitochondria; in one experiment a 30% inhibition of state 3 respiration was observed with 4.2 mM-fluorocitrate added to mitochondria in state 1, i.e.

at an [inhibitor]/[substrate] molar ratio $1:4.8 \times 10^5$. Respiration uncoupled from phosphorylation by 0.1 mM-2,4-dinitrophenol was inhibited by fluorocitrate to the same degree as was coupled state 3 respiration.

Fig. 1. Oxygen-electrode traces of the effect of fluorocitrate on oxidation of citrate by rat liver mitochondria. The reaction medium contained in 3.6 ml. final volume: 10 mM-sodium-potassium phosphate buffer, pH 7.4; 10 mM-tris-chloride buffer, pH 7.4; 100 mM-NaCl; 10 mM-MgCl₂; 14 mM-sucrose; 8.3 mg. of mitochondrial protein. The mitochondrial suspension was added to the medium and the mixture equilibrated at 25° for 1 min. before the recording of the oxygen uptake. The arrows indicate the additions of: A, 2 mM-citrate; B, 2 μmoles of ADP; C, 6.7 μM-fluorocitrate; The [inhibitor]/[substrate] molar ratio was 1:300. Control (curve IV): respiratory control index, 5.0; oxygen uptake in state 3, 32.4 μl./mg. of mitochondrial protein/hr.; ADP/O ratio, 2.11.

Fig. 2. Effect of fluorocitrate concentration on the inhibition of oxidation of citrate by rat liver mitochondria. Experimental details were as described in Fig. 1. Fluorocitrate was added to mitochondria before citrate or ADP (respiratory state 1). V , Rate of uptake of oxygen (μl./mg. of mitochondrial protein/hr.) of control in respiratory state 3; V_4 , rate of uptake of oxygen in the presence of fluorocitrate.

Table 1. Effects of fluorocitrate on oxidation of citrate and cis-aconitate by rat liver mitochondria

Experimental details were as described in Fig. 1. Fluorocitrate was added to the mitochondria in respiratory state 4 and 750 nmoles of ADP were added 1 min. later.

Additions	O ₂ uptake (μl./mg. of mitochondrial protein/hr.)		Respiratory control index	ADP/O ratio	Inhibition of oxidation (%)
	State 4	State 3			
1 mM-Citrate	6.4	25.6	4.0	2.58	—
1 mM-Citrate + 0.1 mM-fluorocitrate	8.0	8.0	1.0	—	100
1 mM-cis-Aconitate	7.2	36.0	5.0	2.78	—
1 mM-cis-Aconitate + 0.1 mM-fluorocitrate	8.0	36.0	4.5	2.78	0
1 mM-Citrate + 1 mM-cis-aconitate	6.4	41.6	6.5	2.88	—
1 mM-Citrate + 1 mM-cis-aconitate + 0.1 mM-fluorocitrate	5.6	43.2	7.7	2.78	0

block (Fig. 3, curve I), whereas the addition of 10mM-*cis*-aconitate caused a slight reversal of the inhibition (Fig. 3, curve II). On the other hand, when 0.1mM-fluorocitrate was added to mitochondria in state 4 and the substrate was 1mM-citrate, the subsequent addition of 10mM-*cis*-aconitate caused a moderate reversal of the inhibition of the enzyme (Fig. 3, curve III). This reversal suggests that this inhibition may be of the competitive type. Finally, the addition to mitochondria in state 1 of 1mM-*cis*-aconitate, 0.1mM-fluorocitrate and 10mM-citrate at 1 min. intervals, followed by ADP (Fig. 3, curve IV), resulted in a respiratory control index, oxygen uptake rate and ADP/O ratio similar to those observed when *cis*-aconitate alone was added to mitochondria. This indicates that the affinity of *cis*-aconitate for the mitochondria-bound aconitate hydratase is much higher than that of citrate.

Effect of fluorocitrate on aconitate hydratase found in the cell sap and on the enzyme solubilized from mitochondria. The aconitate hydratases found in the cell sap and extracted from mitochondria were not subjected to any purification procedure, in order to avoid possible structural changes. The extramitochondrial enzyme preparations were devoid of cytochrome oxidase activity, indicating that insignificant disruption of mitochondria had

occurred in the homogenization and centrifugation procedures.

The aconitate hydratase preparations obtained from mitochondria were not contaminated by mitochondrial inner-membrane fragments since they also did not show detectable cytochrome oxidase activity. Recovery experiments demonstrated

Table 2. *Distribution of aconitate hydratase activity in cell fractions of rat liver*

Experimental details were as described in the Materials and Methods section.

Fractions	Distribution of activity	
	(ΔE_{240} /g. wet wt. of tissue/min.)	(%)
Tissue debris and nuclei	0.46	9
Mitochondria	1.13	22
Cell sap	3.46	69

Fig. 3. Oxygen-electrode traces of the effects of citrate and *cis*-aconitate on the inhibition of mitochondria-bound aconitate hydratase by fluorocitrate. Experimental details were as described in Fig. 1. The arrows indicate the additions of: A, citrate, 10mM in Expts. I and IV, and 1mM in Expt. III; B, *cis*-aconitate, 10mM in Expts. II and III, and 1mM in Expt. IV; C, 750nmoles of ADP; D, 0.1mM-fluorocitrate. Mitochondrial protein was 12.6mg. In the experiment depicted by curve IV: respiratory control index, 4.4; oxygen uptake 21.3 μ l./mg. of mitochondrial protein/hr.; ADP/O ratio 2.34.

Fig. 4. Inhibition by fluorocitrate of aconitate hydratase solubilized from rat liver mitochondria. Incubation mixtures (3ml. final volume) contained 50mM-sodium-potassium phosphate buffer, pH 7.4, and 30mM-citrate or 10mM-DL-isocitrate; the reaction was started by the addition of 0.05–0.10 ml. of enzyme preparation. Readings at 240nm. were taken at 30sec. intervals; after 2min. (arrow) 10 μ l. of 0.15M-fluorocitrate was added. 0.5mM-Fluorocitrate in 3.0ml. of 50mM-phosphate buffer, pH 7.4, caused an E_{240} change from 0.070 to 0.100; this value was subtracted from the actual readings. ●, DL-Isocitrate; 130 μ g. of protein/ml. ○, Effect of 0.5mM-fluorocitrate on the conversion of D-isocitrate into *cis*-aconitate. Inhibition: 55%. ■, Citrate; 113 μ g. of protein/ml. □, Effect of 0.5mM-fluorocitrate on conversion of citrate into *cis*-aconitate. Inhibition, 69%.

Fig. 5. Inhibition by fluorocitrate of aconitate hydratase found in the cell sap of rat liver. Experimental details were as described in Fig. 4. Fluorocitrate was added at the time indicated by the arrow. ●, DL-Isocitrate; 150 μ g. of protein/ml. ○, Effect of 0.5 mM-fluorocitrate on conversion of D-isocitrate into *cis*-aconitate. Inhibition: 22%. ■, Citrate; 150 μ g. of protein/ml. □, Effect of 0.5 mM-fluorocitrate on conversion of citrate into *cis*-aconitate. Inhibition, 29%.

Fig. 6. Inhibition by fluorocitrate of aconitate hydratase solubilized from rat liver mitochondria. Experimental details were as described in Fig. 4. ●, 30 mM-Citrate; ○, 10 mM-citrate; □, 3 mM-citrate.

that about 95% of the enzyme activity was in the first water extract. The second extract, obtained by centrifugation of the mitochondrial solid residue previously resuspended in water at 0°, contained residual 5% of the enzyme solubilized by the freezing-and-thawing procedure. However, the insoluble residue tested by the method of Morrison (1954) could still catalyse the formation

Fig. 7. Inhibition by fluorocitrate of aconitate hydratase of the rat liver cell sap. Experimental details were as described in Fig. 4. ●, 4.0 mM-Citrate; ○, 2.5 mM-citrate; □, 2.0 mM-citrate.

of citrate from *cis*-aconitate, although this activity accounted for only about 6% of the total present in the original mitochondrial fraction. No attempts were made to solubilize all the enzyme contained in the mitochondria. The pH optima and Michaelis constants of the enzymes have not been investigated in detail.

Distribution experiments showed that 70–80% of the rat liver aconitate hydratase activity occurred in the cell sap and the remaining 20–30% was associated with the mitochondria. The results of a typical experiment are shown in Table 2; they are in agreement with the results obtained by Dickman & Speyer (1954).

The effect of fluorocitrate on the two enzyme preparations was tested both on the citrate $\rightarrow$ *cis*-aconitate and the isocitrate $\rightarrow$ *cis*-aconitate reactions and the results of typical experiments are shown in Figs. 4 and 5. The inhibition was of the competitive type for both enzymes (Figs. 6 and 7).

Since the inhibition was competitive and the substrate/inhibitor concentration ratio in the assays of Fig. 4 was 60:1 for [citrate]/[fluorocitrate] and 10:1 for [D-isocitrate]/[fluorocitrate], it was apparent that the reaction citrate $\rightarrow$ *cis*-aconitate was inhibited by fluorocitrate more strongly than the reaction isocitrate $\rightarrow$ *cis*-aconitate. By carrying out the test on the two substrates at equal concentrations the difference in sensitivity of the two reactions to fluorocitrate was evident (Table 3). The results of this experiment confirmed also that the enzyme activity extracted from the mitochondria was more sensitive to the inhibitory action of fluorocitrate than was the enzyme activity found in the cell sap; fluorocitrate concentrations of 0.3 mM and 1.2 mM caused 50% inhibition of the

Table 3. *Inhibition by fluorocitrate of rat liver aconitate hydratase solubilized from mitochondria and found in the cell sap*

Experimental details were as described in the Materials and Methods section and in Fig. 4.

Enzyme	Substrate	10 ³ × Sp. activity (ΔE ₂₄₀ /mg. of protein/min.)		Inhibition (%)
		Control	Fluorocitrate (0.5 mM)	
Solubilized from mitochondria	10 mM-Citrate	80	33	59
	20 mM-DL-Isocitrate	200	143	29
Found in the cell sap	10 mM-Citrate	53	43	19
	20 mM-DL-Isocitrate	84	84	0

Table 4. *Effect of poisoning in vivo with fluoroacetate and fluorocitrate on rat liver aconitate hydratase solubilized from mitochondria and found in the cell sap*

Experimental details were as described in the Materials and Methods section and in Fig. 4. Means ± s.e.m. are given. The numbers in parentheses are the numbers of experiments. Citrate or isocitrate was used as substrate.

Material injected into rats	10 ³ × Sp. activity of aconitate hydratase (ΔE ₂₄₀ /mg. of protein/min.)							
	Solubilized from mitochondria				Found in the cell sap			
	Citrate (30 mM)	Δ (%)	DL-Isocitrate (10 mM)	Δ (%)	Citrate (30 mM)	Δ (%)	DL-Isocitrate (10 mM)	Δ (%)
Saline	80 ± 3.7 (4)	—	169 ± 24.6 (4)	—	64 ± 3.7 (5)	—	102 ± 3.7 (5)	—
Fluoroacetate (10 mg./kg. body wt./hr.)	103 ± 10.4 (3)	+29	215 ± 15.2 (3)	+27	60 ± 1.5 (5)	0	107 ± 6.6 (5)	0
Fluorocitrate (40 mg./kg. body wt./hr.)	95 ± 4.9 (3)	+19	215 ± 6.5 (3)	+27	50 ± 3.1 (5)	-22	84 ± 10.7 (5)	-18

respective enzymes when catalysing the reaction citrate → *cis*-aconitate.

Experiments in vivo. Previous experiments (P. Buffa & V. Guarriera-Bobyleva, unpublished work) showed that liver mitochondria isolated from fluoroacetate- and fluorocitrate-poisoned rats were inhibited in their ability to oxidize added citrate but not *cis*-aconitate, and the results of the experiments on the effects of fluorocitrate on the oxidation of citrate and *cis*-aconitate by normal rat liver mitochondria *in vitro*, reported above, confirmed and extended these findings. To gain a better knowledge on the mode of action of fluoroacetate in the liver cell, rats were injected with fluoroacetate or fluorocitrate and the activities of aconitate hydratase solubilized from mitochondria and of the enzyme found in the cell sap were measured. The results of these experiments (Table 4) show that the activity of the aconitate hydratase solubilized from mitochondria was not only not inhibited but was significantly increased both in fluoroacetate and fluorocitrate poisoning. Fluoroacetate poisoning caused no effect on the aconitate hydratase found in the cell sap, whereas

fluorocitrate poisoning resulted in a slight decrease of this enzyme activity.

DISCUSSION

The results of the present study confirmed and extended the findings of Dickman & Speyer (1954) and of Shepherd *et al.* (1955) that by differential centrifugation of liver homogenates it is possible to separate two aconitate hydratase activities, one associated with the mitochondrial fraction and one associated with the cell-sap supernatant. This finding raises the question whether the activity found in the cell sap leaks out from the mitochondria. This appears rather improbable. Isolated mitochondria can be repeatedly washed with 0.25 M-sucrose without releasing any of their aconitate hydratase activity, and to obtain the enzyme in soluble form the mitochondria have to be suspended in water and frozen and thawed, or disintegrated with ultrasonic vibrations. Our preparations of cell sap did not contain cytochrome oxidase activity and this indicated that the mitochondria had suffered little damage during the

homogenization of the liver tissue. Thus the present evidence points to a dual localization of aconitate hydratase in the liver cell.

When bound to mitochondria aconitate hydratase appears to have a higher sensitivity to added fluorocitrate than does the extracted enzyme. The question then arises whether this may be due to an accumulation of fluorocitrate within the mitochondria. This possibility cannot at present be excluded, although preincubation of mitochondria (in the presence of 2mM-citrate) for up to 3min. with 10 μ M-fluorocitrate does not give increased inhibition. This behaviour of mitochondrial aconitate hydratase is similar to that exhibited by dinitrophenol-stimulated adenosine triphosphatase: when the enzyme is bound to the mitochondrion it is inhibited by oligomycin, whereas the solubilized enzyme is not affected by this inhibitor (Racker, 1963).

The conversion of citrate into *cis*-aconitate is inhibited much more strongly than the conversion of *cis*-aconitate into isocitrate. This provides an explanation of the observation that the mitochondria isolated from the liver of a fluoroacetate-poisoned rat show an inhibition of oxidation of citrate but not of oxidation of *cis*-aconitate (P. Buffa & V. Guarriera-Bobyleva, unpublished work).

No clear evidence is available on the type of inhibition by fluorocitrate of the mitochondria-bound aconitate hydratase, although it was observed that *cis*-aconitate could partly restore the respiration of the inhibited mitochondria. On the other hand inhibition by fluorocitrate of the enzyme solubilized from mitochondria was clearly competitive and reversible.

The results reported from purification, kinetic, inhibition and pH studies are consistent with the hypothesis that aconitate hydratase consists of a single protein with one active centre (Dickman, 1961). However, Peters & Shorthouse (1969), using a soluble pig heart preparation, found that storage at 0° largely inactivates the citrate $\rightarrow$ *cis*-aconitate reaction but has little effect on the isocitrate $\rightarrow$ *cis*-aconitate reaction; this suggests the existence of two aconitate hydratases (or active centres) of different specificity. The effects of fluorocitrate may be explained both by the one-enzyme or by the two-enzymes (or two-active-centres) hypotheses. In the former case the results would be explained by a differential affinity of citrate, *cis*-aconitate and fluorocitrate for the enzyme: fluorocitrate would have an affinity for the mitochondria-bound enzyme a little higher than that of *cis*-aconitate and much stronger than that of citrate. The two-enzymes hypothesis would imply that the enzyme catalysing the reaction citrate $\rightarrow$ *cis*-aconitate is strongly inhibited by fluorocitrate whereas the enzyme catalysing the reaction *cis*-aconitate $\rightarrow$

isocitrate is only slightly sensitive to the inhibitor.

In conclusion, under our conditions fluorocitrate appears to be a potent inhibitor of mitochondria-bound aconitate hydratase and a highly specific one for the reaction citrate $\rightarrow$ *cis*-aconitate, whereas it appears less inhibitory and less specific for the enzyme solubilized from the mitochondria.

Fluorocitrate causes a pattern of inhibition of the aconitate hydratase of the cell sap similar to that observed for the enzyme solubilized from mitochondria, but the inhibitory effect is still less pronounced. It is not clear whether this lower sensitivity reflects a real difference in the enzyme molecule or whether it depends on environmental factors. Our results cannot be compared with those obtained by Peters (1961) on pig heart soluble aconitate hydratase because our soluble preparations were obtained from rat liver and were very crude; however, our findings are similar to those obtained by Fanshier, Gottwald & Kun (1962) using synthetic fluorocitrate and a soluble preparation of aconitate hydratase from rat kidney mitochondria.

As is well known (Peters, 1952), fluoroacetate *in vivo* is converted into fluorocitrate, which then inhibits the mitochondria-bound aconitate hydratase and blocks oxidation of citrate. The extramitochondrial enzyme of the cell sap is not affected by fluoroacetate poisoning presumably because the fluorocitrate formed within the mitochondria remains firmly bound, perhaps to the mitochondrial aconitate hydratase. The present findings in liver confirm the observation by Buffa & Peters (1949) that a soluble preparation of aconitate hydratase obtained from the hearts of rats poisoned with fluoroacetate showed a normal activity.

It is noteworthy that fluorocitrate *in vivo* inhibited the extramitochondrial aconitate hydratase only to a moderate extent, leaving a large proportion of active enzyme available. The paradoxical increase of specific activity of the enzyme solubilized from the mitochondria cannot be explained at present; however, Buffa, Muscatello, Godina & Barasa (1964) observed that fluoroacetate poisoning provokes mitochondrial swelling, and it may be that more enzyme can be extracted relative to inactive proteins.

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